Biodiversity and Biomedicine

Our Future

Edited by
Munir Ozturk, Dilfuza Egamberdieva,
and Milica Pešić

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Foreword

Prof. Dr. Zabta Khan Shinwari
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Biodiversity and Biomedicine: Our Future is a well thought-out title. Climate change and unsustainable use of natural resources warranted such efforts. The worry is that loss of biodiversity is not only because of habitat loss, alien species, pollution, and overexploitation but also because of frontier technologies. This is evident from the fact that "a century ago, only 15% of Earth's surface was used to grow crops and raise livestock. Today, more than 77% of land (excluding Antarctica) and 87% of the ocean has been modified by the direct effects of human activities" (Nature, October 31, 2018). Biodiversity loss may accelerate the spread of infectious diseases.

The connection between humans and biodiversity dates back to the beginning of human life on this planet. Natural resources, like plants, microbes, and endophytic microorganisms are a potential source of bioactive chemical entities which possess tremendous value in agriculture, biotechnology, biomedicine, and pharmaceutical industries. There is an ever increasing requirement for the search for novel bioactive ingredients from natural sources which can be used in flavor, fragrance, food, and pharmaceutical industries. Disease-causing pathogens are developing resistance to the existing reservoir of therapeutants at a rapid rate. In this age of antibiotic resistance, the discovery of new lead compounds is a desperate need. Naturally occurring bioactive compounds isolated from different organisms show promise in this regard. Natural product research has led to many success stories. Most of the anticancer medicines were first discovered in plants. Compounds like vinblastine, vincristine, and taxol, which are popular in chemotherapies, were first isolated from *Catharenthus roseus* and *Taxus baccata.* Silymarin, isolated from *Silybum marianum*, is currently prescribed in hepatitis. The isolation of artemisinin, from sweet wormwood (*Artemisia annua*), led to the award of the Nobel Prize. These developments are not restricted only to the plant kingdom. In the past decades an increasing number of bioactive compounds of animal origins have been identified and extracted for human use as dietary and therapeutic supplements helping to cure various diseases. A classic example of how the animal kingdom provides solutions to our health issues is given by honey from bees. Venoms are also sources for the isolation of different bioactive compounds that can be used in different applications. Researchers are now beginning to explore marine resources for bioactive chemical entities. All these developments point toward the caring role of Mother Nature.

In this context, the aim of the current volume, *Biodiversity and Biomedicine: Our Future* is to offer an updated perspective regarding the advances and current status of natural products research. This book brings together eminent scientists who have contributed comprehensive chapters in their respective fields under the umbrella of Biodiversity and Biomedicine. The importance and future of the bioactive compounds from natural resources has been discussed in detail. The present volume will be of interest to scientists, academics, the pharmaceutical, biotechnological, and industrial sectors, and to all those who are interested in natural products.

The book encompasses all important aspects of biomedicine, for example, the plant microbiome as a source of biological active compounds is in fact very important because only a few of these plants have ever been completely studied relative to their endophytic biology. These microbes are threatened by extinction with the loss of plant diversity (partly due to climate change) and need to be discovered before they are extinct.

The topics of chemodiversity in natural plant populations and plant biodiversity in health and medicine are significant, as can be measured from the fact that the market of nutraceuticals obtained from natural resources is forecast to be US$ 340 billion by 2024. At the same time care has to be taken of endangered species that are reputed (falsely) to possess medicinal properties. Taxol is an example of the synergy of endophytic microorganisms with plants that can be used in the treatment of chronic diseases like cancer. One more such example is that of *Fagonia* which is effective in breast cancer.

I congratulate the authors, such books are not only a great addition to the knowledge base but also will bring readers to the libraries.

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Preface

Life on Earth depends on biodiversity as it maintains homeostasis which is essential for all ecosystems and includes organisms that provide primary production as well as organisms responsible for decomposition. Humankind is a part of Earth's biodiversity and we depend on it to satisfy all our needs for survival. The total number of recorded living species is estimated at 1.5 million. During the last century, the unsustainable use of natural resources has damaged it so severely that this situation now threatens humankind. Nearly 5000 taxa of plants are said to have become extinct during the last 2 centuries and 17 plants are lost every year. The rate at which species are becoming extinct due to the increasing and prevailing needs of just one species (*Homo sapiens*) is much higher than the natural occurring rate. The extinctions have a direct impact on human society. Finding useful drugs in wild biota can be a mixed blessing. Among about 1000 antibiotics now known are, only mentioning the most famous: penicillin obtained from *Penicillium chrysogenum*, cephalosporin C from *Cephalosporium acremonium*, griseofulvin from *Penicillium griseofulvum*, bacitracin, chloromycin, erythromycin, streptomycin, and tetracycline from various bacteria, and mimosamycin from nudibranch. In addition, anticancer drugs such as cantharidin is obtained from the Chinese blister beetle, podophyllotixin is obtained from *Podophyllum peltatum*, monocrotaline is obtained from *Crotalaria spectabilis*, vincristine−vinblastine is obtained from rosy periwinkle, and taxol comes from the yew (*Taxus brevifolia*). Similarly aspirin, codeine, morphine, cocaine, and tetrodotoxin are used as painkillers and obtained from willow, opium poppy, *Erthroxylum coca*, and a Central American frog, respectively. Digitoxin is used as a heart stimulant and obtained from Foxglove, while atropine is used as a pupil dilator and obtained from deadly nightshade (also known as belladona).

We do not know much about the value systems of our successors. Perhaps they will need vast quantities of some species, considered insignificant or even harmful by us presently. We have some options: sustainable development requires human beings to raise and improve their quality of life in harmony with nature and conserve the balance of the ecosystems which supply the fundamental support to sustain our lives.

This edited volume *Biodiversity and Biomedicine: Our Future* provides different evidence for the biomedical values of Earth's species: from traditional use of plants, fungi, microbes, marine organisms, insects, amphibians, and reptiles to the most sophisticated scientific approaches for the identification of their bioactive components. We have not completely explored the molecular diversity of our biota. Some of our book authors have identified this problem in their published viewpoints: "Traditional knowledge about their usage is depleting, while modern society is not sufficiently aware that drug discovery from natural sources has always been, and will continue to be critical for most, if not all, aspects of health care."

This book aims to raise awareness about incredible but understudied biodiversity with respect to its biomedical potential. Even human biodiversity needs to be explored more fully to enable the personalized therapeutic approach. It is in our interests to ensure human health as well as the safety of other species because planetary health relies on rich biodiversity. The loss of biodiversity decreases our ability to discover new products, metabolites, and genes that are beneficial for human health.

This volume includes 26 chapters from Albania, Australia, Germany, India, Indonesia, Malaysia, Mauritius, Montenegro, Oman, Pakistan, Philippines, Poland, Russian Federation, Saudi Arabia, Serbia, South Africa, Turkey, the United Kingdom, the United States, and Uzbekistan. All chapters were written by experts from different research fields who are capable of associating ethnobotany with new bioactive components isolated from traditional healing sources, identifying specific biological activity of naturally isolated principles in terms of anticancer, antibiotic, antiviral, antifungal, antidiabetic, and other effects, and importantly providing scientific advice and recommendations for biodiversity preservation for future biomedical discoveries. Finally looking into biodiversity as an irreplaceable resource for the biomedical breakthroughs, the authors associate the availability of species for medical research with human survival and well-being.

The 26 chapters included in this volume are titled as: Plant Microbiome: Source for Biological Active Compounds; Chemodiversity in Natural Plant Populations as a Base for Biodiversity Conservation; Harnessing the Potential of Plant

Biodiversity in Health and Medicine: Opportunities and Challenges; Biomining Fungal Endophytes from Tropical Plants and Seaweeds for Drug Discovery; Biomedicine Developments Based on Marine Biodiversity: Present and Future; Superbugs, Silver Bullets and new Battlefields; The Benefits of Active Substances in Amphibians and Reptiles and the Jeopardy of Losing Those Species Forever; Human Genetic Diversity in Health and Disease; Potential for Cancer Treatment: Natural Products from the Balkans; Biodiversity of Wild Fruits with Medicinal Potential in Serbia; Botanicals from the Himalayas with Anticancer Potential—An Emphasis on Kashmir Himalayas; Diversity and Bioprospect Significance of Macrofungi in the Scrub Jungles of Southwest India; Mushroom and Plant Extracts as Potential Intervening Supplements in Diabetes Management; Anticancer Activities of Marine Macroalgae: Status and Future Perspectives; Insights into the Bioactive Compounds of Endophytic Fungi in Mangroves; Essentiality of Mint: Current Understanding and Future Prospects; *Azadirachta indica*: The Medicinal Properties of The Global Problems-Solving Tree; Advancements in Plant Transgenomics Approach for the Biopharmaceutics and Vaccines Production; Secondary Metabolites from Endangered *Gentiana*, *Gentianella*, *Centaurium*, and *Swertia* species (Gentianaceae): Promising Natural Biotherapeutics; Grape (*Vitis vinifera* L.): Health Benefits and Effects of Growing Conditions on Quality Parameters; Flavonoids in Cancer Therapy: Current and Future Trends; Personalized Biomedicine in Cancer: From Traditional Therapy to Sustainable Healthcare; Tumor-Specific Genetic Profiling and Therapy in Biomedicine; Vascular and Bone Marrow Explant Models to Assess In Vitro Hematotoxicity of Herbal Extracts; Nature-Inspired Synthetic Analogues of Quorum Sensing Signaling Molecules as Novel Therapeutics against *Pseudomonas aeruginosa* Infections; and Biomedicine: Biodiversity's Panacea? Context of Commodification.

Many communities rely on natural products collected from ecosystems for medicinal and cultural purposes, in addition to their use as food. Although synthetic medicines, many of which were synthesized according to the chemical structures found in nature, are available for many purposes, the global need and demand for natural products persists. In addition, biomedical research relies on plants, animals, and microbes to understand human physiology and pathological conditions, as well as to prevent and treat human diseases. Human activities on Earth alter the interactions among organisms and their environment. Patterns of infectious diseases reservoirs and transmission are particularly sensitive. Still, a healthy biodiversity provides quite a lot of services for humankind.

Biodiversity loss can have significant direct human health impacts if ecosystem services are no longer adequate to meet social needs. Additionally, the biophysical diversity of microorganisms, flora, and fauna provides extensive knowledge which carries important benefits for biological, health, and pharmacological sciences. Significant medical and pharmacological discoveries are made through greater understanding of the Earth's biodiversity. Keeping the facts cited above in view, there is a great need to prepare detailed inventories very quickly; sustainable and alternative methods/approaches should be developed for the species gathered from nature; situation assessments made, data banks established; and data management models improved. Therefore this book will be an important literary source for scientists, students, teachers, medical doctors, pharmacists, policy makers, as well as for local communities of bio-rich countries who want to learn about the molecular diversity of the different species that present the richest reservoir for future discoveries in medicine.

Munir Ozturk, Dilfuza Egamberdieva and Milica Pešić

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Chapter 1

Plant microbiome: source for biologically active compounds

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1.1 Introduction

Medicinal plants are known as a source for biologically active metabolites with therapeutic potential and have been used worldwide since ancient times for the treatment of a number of diseases, such as hepatic and cardiovascular pain, inflammation and inflammatory-mediated pain, asthma, gastrointestinal symptoms, skin disease, and urinary problems (Ozturk & Hakeem, 2018, 2019a, 2019b; Tian et al., 2014). According to the World Health Organization, medicinal plants are commonly used in developing countries to treat many kinds of disorders, and traditional medicine has an economic importance as well (WHO, 2013).

In many developing countries herbaceous flora and medicinally important trees are not well studied and new drugs could be developed from them for health benefits. The use of synthetic drugs and antibiotics has been reported to be hazardous due to side effects. Moreover, there is concern regarding multidrug resistance microbes, and as such new alternative natural drugs should be developed (Compean & Ynalvez, 2014). The use of medicinal plants for the treatment of many kinds of diseases has become popular (Bharti, Bai, Seasotiya, Malik, & Dalal, 2012).

Plant-derived products used in old medical treatments have increased our current knowledge of herbal medicines (Sarker & Nahar, 2007). The bark, roots, leaves, flowers, and fruits of *Sambucus nigra* have been used widely in the Mediterranean regions for the treatment of various ailments (Valles, Angels, & Agelet, 2004). Similarly, plants like *Buxus papillosa*, *Peganum harmala*, and *Solanum surattense* have been used as a diaphoretic, purgative, antirheumatic, analgesic, and aphrodisiac (Ahmad, Ahmad, & Jan, 2002). The plant extracts of *Achyranthes aspera* and *Lantana camara* have demonstrated antimicrobial activity against various microbes pathogenic to humans (Gupta, Kartik, Manoj, Singh, & Alka, 2010). Similar observations have been reported by Guesmi, Ben Hadj, and Landoulsi (2017), who noted that a methanol extract of *Lavandula multifida* exhibited high antibacterial activity against *Bacillus cereus*. The extracts of *Punica granatum* and cumin have given positive tests against human pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi* (Duan, Li, & Gao, 2013; Mahboubi, Asgarpanah, Sadaghiqani, & Faizi, 2015; Mishra et al., 2017).

Plants synthesize an extremely diverse range of chemical compounds and represent a great potential for the discovery and development of new pharmaceuticals (Edeoga, Okwu, & Mbaebie, 2005; McChesney, Venkataraman, & Henri, 2007). The biologically active compounds synthesized by microbes help plants to survive and flourish in hostile environments (Cushnie, Cushnie, & Lamb, 2014; Egamberdieva & Teixeira da Silva, 2015; Egamberdieva, Wirth, Behrendt, Parvaiz, & Berg, 2017).

There are many reports on plant secondary metabolites with antibacterial and antifungal properties (Egamberdieva & Teixeira da Silva, 2015). Biological active compounds such as tannins, alkaloids, phenolic compounds, flavonoids, and terpenes found in plant root systems, rhizomes, stems, flowers, and fruits are valuable as pharmaceuticals and nutraceuticals (Palombo, 2006). Plant-derived chemicals show a wide range of biological effects; for example, polyphenols (phenolic acid, and flavonoids) show anticarcinogenic, antimutagenic, antimicrobial, and antiinflammatory effects (Ofokansi, Esimone, & Anele, 2005; Okwu, 2004).

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The medicinal plants synthesize different biologically active metabolites, such as saponins, flavonoids, and alkaloids (David, Elumalai, Sivakumar, Therasa, & Thirumalai, 2010; Lacaille-Dubois & Wagner, 1996; Omulokoli, Khan, & Chhabra, 1997). These are responsible for the antimicrobial activity of plant extracts. There are many reports on the phytochemical and biological properties of medicinal plants as well as their metabolites (David et al., 2010; Egamberdieva & Jabborova, 2018; Gnat et al., 2017). For example, *Ziziphora capitata* and *Hypericum perforatum* contain essential oils, flavanoids, and sterols that are known as remedies for infectious disease (Sonboli, Mirjalili, Hadian, Ebrahimi, & Yousefzadi, 2006). *H. perforatum* contains essential oils, tannins, flavonoids, xanthones, and hyperforin that are used for the treatment of skin disorders, diarrhea, and hepatic diseases (Zhaparkulova, Srivedavyasasri, Sakipova, & Ross, 2015). Phytochemicals, including saponins, flavonoids, phenolics, and glycosides, have been reported from the seeds of *Piliostigma thonningii*. These show antimicrobial activity against many human pathogenic bacteria, including *E. coli* and *Bacillus subtilis* (Jimoh & Oladiji, 2005). Many researchers have reported the antimicrobial activity of flavonoids, tannins, sterols, and essential oils (Akbar & Al-Yahya, 2011; Al-Yahya, Al-Meshal, Mossa, Al-Badr, & Tariq, 1990). The essential oils from *Blepharis cuspidata* and *Thymus schimper* are reported to exhibit antimicrobial activity against several human pathogenic bacteria including *S. aureus* (Gadisa et al., 2019). The root and bark of *Periploca laevigata* shows antioxidant activity and antagonism against various fungi and bacterial species (Hajji et al., 2019).

The traditional use of medicinal plants has been validated by a large number of studies where the phytochemicals present in active extracts have been investigated. However, the microbes associated with medicinally valuable plants and their metabolic activities have not been evaluated fully, and few scientific studies have been reported. The inter- and/or intracellularly residing endophytic microbes within medicinally important plants have proven to be potential sources of drug discovery (Egamberdieva et al., 2011, 2015; Venugopalan & Srivastava, 2015). Plant physiology and chemical composition in plant exudates strongly affect the microbial communities inside the plant tissue (Chaparro, Badri, & Vivanco, 2014; Köberl et al., 2013). Their diversity and physiological features are highly affected by the genetic background of host plants, ecological habitats, and the phytoconstituents. Secondary metabolites of diverse pharmacological activities are produced by endophytic bacteria, some of these metabolites are the same as those produced by their host plants (Cho et al., 2015; Egamberdieva & Teixeira da Silva, 2015). Therefore these are considered as potential sources of biologically active compounds with higher therapeutic potential than their hosts (Gouda, Das, & Sen, 2016).

1.2 Diversity of endophytic bacteria

Endophytic microbes of medicinal plants live inside the plant tissue and produce the same metabolites within the living tissues of the host plant (Dos Santos et al., 2016; Passari, Mishra, Saikia, Gupta, & Singh, 2015; Verma et al., 2009). Kaewkla and Franco (2013) have reported *Streptomyces* species colonizing Australian native trees. The root of *Aloe vera* is colonized with 13 microbial genera, including *Aeromonas*, *Bacillus*, *Enterobacter*, *Chryseobacterium*, *Cedecea*, *Cronobacter*, *Klebsiella*, *Macrococcus*, *Pseudomonas*, *Pantoea*, *Providencia*, *Sphingobacterium*, and *Shigella*. Among these the dominant genera found are *Bacillus* and *Pseudomonas* (Akinsanya, Goh, Lim, & Tinga, 2015). The endophytic actinobacteria are also found in *A. vera*, and *Mentha arvensis*, and include *Actinopolyspora* sp., *Micromonospora* sp., *Saccharopolyspora* sp., and *Streptomyces* sp. (Gangwar, Dogra, Gupta, & Kharwar, 2014). The microbes belonging to *Streptomyces* are common in plant tissues of the medicinal plants *Leucas ciliata* and *Rauwolfia densiflora* (Akshatha, Nalini, D'Souza, & Prakash, 2014) (Table 1.1).

The roots of *Codonopsis lanceolata* colonized with endophytic bacteria belong to *Bacillus polyfermenticus*, *B. subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus* (Su, Kang, Cao, Mo, & Hyde, 2014). Among microbial isolates *B. subtilis*, *B. pumilus*, and *B. licheniformis* show antifungal activity against plant pathogenic fungi such as *Phytophthora capsici*, *Fusarium oxysporum*, and *Rhizoctonia solani*.

Singh, Kumar, Singh, and Pandey (2017) have reported endophytic microbes colonizing plant tissues of *Dracaena cochinchinensis* L. They belong to *Arthrobacter*, *Brevibacterium*, *Brachybacterium*, *Microbacterium*, *Nocardioides*, *Pseudonocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella*. In another study *Paenibacillus polymyxa* has been found in the leaf tissue of *Panax ginseng* and the strain is able to stimulate plant growth, as well as increase ginsenoside concentration (Gao et al., 2015).

The medicinal plant *Panax notoginseng* is colonized by several bacterial genera including *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* (Tan & Zou, 2001). Checcucci et al. (2017) have observed that there is a high taxonomic diversity of microbes in the leaves, roots, and rhizospheric soil of *Thymus* spp. The actinomycete species with high antimicrobial activity are common in the plant tissue of 300 plants

TABLE 1.1 Diversity of endophytic microbes from medicinal plants.

Medicinal plant	Species	Reference
Aloe vera, *Ocimum* (*tulsi*), *Mentha arvensis*, *Ocimum sanctum*	*Cladosporium* sp., *Nigrospora* sp. *Aspergillus* sp., *Penicillium* sp., *Gliocladium roseum*, *Actinopolyspora* sp., *Micromonospora* sp., *Saccharopolyspora* sp., *Streptomyces* sp.	Akinsanya et al. (2015); Gangwar et al. (2014)
Dracaena cochinchinensis	*Rhodococcus*, *brevibacterium*, *Nocardioides*, *Microbacterium*, *Nocardiopsis*, *Brachybacterium Tsukamurella*, *Arthrobacter*, *Pseudonocardia*	Singh et al. (2017)
Panax ginseng	*Paenibacillus polymyxa*, *Bacillus altitudinis*	Gao et al. (2015); Tan and Zou (2001); Song et al. (2017)
Thymus vulgaris, *T. citriodorus*	*Pseudomonas*, *Enterobacteriacea*	Checcucci et al. (2017)
Siparuna crassifolia, *Calycophyllum acreanum*, *Capirona decorticans*, *Ocotea longifolia*, *Aspidosperma* sp., *Palicourea longifolia*, *Monstera spruceana*, *Croton lechleri*, *Cantua buxifolia*	*Amycolatopsis* sp., *Micromonospora* sp., *Streptomyces* sp.	Bascom-Slack et al. (2009)
Zingiber officinale, *Alpinia galanga*	*Streptomyces aureofaciens*	Taechowisan and Lumyong (2003)
Hedycium acuminatum	*Fusarium solani*, *F. oxysporum*, *F. semitectum*, *Colletotrichum alienum C. aotearoa*, *C. coccodes*, *C. gloeosporoides*, *Aspergillus parasiticus*	Hastuti et al. (2018)
Leucas ciliate, *Plectranthus tenuiflorus*	*Bacillus* sp., *B. megaterium*, *B. pumilus*, *B. licheniformis*, *Micrococcus luteus*, *Paenibacillus* sp., *Pseudomonas* sp., *Acinetobacter calcoaceticus*	El-Deeb et al. (2013)
Rauwolfia densiflora	*Streptomyces longisporoflavus*, *Streptomyces* sp.	Akshatha et al. (2014)

distributed in the Amazonian rainforest in Peru (Bascom-Slack et al., 2009). Similar observations have been reported by Taechowisan and Lumyong (2003), where actinomycetes, especially *Streptomyces* species associated with *Zingiber officinale* and *Alpinia galangal*, showed the best antimicrobial activity against human and plant pathogenic microbes including *Colletotrichum musae* and *F. oxysporum*. The endophytic fungi isolated from the medicinal plant *Hedycium acuminatum* include species of *Fusarium*, *Colletotrichum*, and *Aspergillus*. These show antimicrobial activity against *Staphylococcus* spp. and *B. cereus* (Hastuti, Asna, & Rahmawati, 2018). Several bacterial isolates, such *Bacillus megaterium*, *Micrococcus luteus*, *Pseudomonas* sp., and *Acinetobacter calcoaceticus*, isolated from *Plectranthus tenuiflorus* produce extracellular enzymes, such as lipase, protease, pectinase, amylase, xylanase, and cellulase. They are also antagonists against human pathogenic microbes such as *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Proteus mirabilis*, and *Candida albicans* (El-Deeb, Fayez, & Gherbawy, 2013). Among the endophytic bacteria associated with *Matricaria chamomilla*, *Calendula officinalis*, and *Solanum distichum*, more antagonists against pathogens have been reported by Köberl et al. (2013). Bafana and Lohiya (2013) have observed high amounts of endophytic bacteria belonging to *Pseudomonas* and *Stenotrophomonas* in the roots of *Origanum vulgare*. *Z. capitata* and *H. perforatum* in Uzbekistan are colonized with endophytic bacteria belonging to the genera *Arthrobacter*, *Bacillus*, *Erwinia*, *Pantoea*, *Serratia*, *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* (Egamberdieva & Teixeira da Silva, 2015). The diversity of endophytic bacteria is higher in *Z. capitata* as compared to *H. perforatum*, indicating the importance of plant phytochemical constituents in selecting the bacterial community. Ahmed, Hassan, El Tobgy, and Ramadan (2014) have found lower microbial populations in the rhizosphere of *M. chamomilla*, but most of the bacteria showed antibacterial activity against pathogenic bacteria. Several studies report on the presence of *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Pantoea* genera in plant tissues of *Tridax procumbens* L. (Preveena & Bhore, 2013), *A. vera* (Akinsanya et al., 2015), and *Andropogon gerardii* Vitman (Rosenzweig, Bradeen, Tu, & Kinkel, 2013).

1.3 Biological activity of endophytic microbes

Plant tissue is a host for microbes that produce various secondary metabolites with biological activity, such as antimicrobials, anticancer agents, antioxidants, and plant growth regulators (Qin, Xing, & Jiang, 2011). A high antagonistic activity against plant pathogens like *Alternaria solani*, *F. oxysporum* f. sp. lycopersici, and *Rhizoctonia* sp. has been observed in the actinobacteria associated with tomato plants (Li, Pan, & Tuo, 2017; Mingma, Pathomaree, & Trakulnaleamsai, 2014). In another study *Streptomyces* sp. has shown antagonistic activity against human pathogenic microbes (Kim, Cho, & Han, 2012), and also against plant pathogenic fungi. Similar observations have been reported by El-Shatoury, El-Karaaly, and El-Kazzaz (2009), where *Achillea fragrantissima*-associated actinomycetes produce antifungal compounds which inhibit the growth of fungal pathogens (Table 1.2). The microbes showing antagonistic activity produce fungal cell wall−degrading enzymes such as chitinases, pectinases, glucanases, and cellulases (Egamberdieva et al., 2011, 2015; Kalai-Grami, Saidi, & Bachkouel, 2014).

Kumar, Kanaujia, and Bafana (2012) have reported the presence of endophytic bacteria with high plant beneficial traits, such as the production of phytohormones, siderophores, and cell wall−degrading enzymes in the plant tissue of *Ajuga bracteosa*.

The endophytic bacteria *Nocardiopsis dassonvillei* produces antimicrobial compounds and inhibits the growth of *Staphylococcus* sp. (Zhang, Lu, & Lu, 2016). Similarly the *P. ginseng*-associated bacteria *Bacillus altitudinis* increases the accumulation of ginsenoside (Song, Wu, Yin, Lian, & Yin, 2017). The endophytic bacteria isolated from *Z. capitata* and *H. perforatum* produce several plant beneficial metabolites, such as indole 3-acetic acid, siderophores, ACC-deaminase enzyme, and cell wall−degrading enzymes that stimulate root systems, improve plant stress tolerance, and protect plants from various plant pathogens (Egamberdieva & Teixeira da Silva, 2015). The majority of bacterial isolates show antagonistic activity against plant pathogenic fungi like *F. oxysporum*, *Fusarium solani*, *Fusarium culmorum*, *Gaeumannomyces graminis*, *Alternaria alternata*, *Botrytis cinerea*, and the oomycete *Pythium ultimum.* In another study actinobacteria from *A. fragrantissima* are reported to have synthesized chitinases or siderophores as well (El-Shatoury et al., 2009).

Nejatzadeh-Barandoz (2013) has found *Pseudomonas* and *Bacillus* species among endophytes associated with *A. vera*. These show antimicrobial activity against human pathogenic bacteria like *S. aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *E. coli.* According to Lin, Zhao, Yang, Guan, and Gong (2013), endophyte-produced biologically active compounds can protect plants from various plant pathogenic insects and pathogens. For example, *B. subtilis* and *B. megaterium* isolated from the medicinal plant *Sophora alopecuroides* reduces *Verticullum* wilt disease of cotton (Lin et al., 2013).

In India, 42 endophytic fungi with antimicrobial activity against potential human pathogens have been isolated from medicinal plants; these include *E. coli*, *S. typhi*, *P. mirabilis*, *S. aureus*, and *Corynebacterium diphtheriae* (Palanichamy, Krishnamoorthy, Kannan, & Marudhamuthu, 2018). The secondary biologically active compound in the endophytic fungi *Alternaria* sp. has been identified as alternariol methyl ether. Similarly, Tejesvi, Kajula, Mattila, and Pirttilä (2011) have isolated endophytic fungi from *Rhododendron tomentosum*—a medicinal plant—with antibacterial (10%) and antioxidant (14%) activities. Nearly 200 fungal isolates belonging to Ascomycota, Basidiomycota, and Mucoromycotina have been studied in medicinal plants like *Camptotheca cuminata*, *Gastrodia elata*, and *Pinellia ternate* (Su et al., 2014). Some of the fungal isolates synthesize biologically active metabolites. The fungal isolates from *C. cuminata* produce camptothecin, and *Colletotrichum gloeosporioides* from *C. cuminata* produces 10-hydroxycamptothecin which are valuable for pharmacological applications. *Actinopolyspora* sp., *Micromonospora* sp., *Saccharopolyspora* sp., and *Streptomyces* sp. synthesize a hydroxamate-type of siderophore, as reported by Gangwar et al. (2014).

The endophytic fungus *Phomopsis* from *Azadirachta indica* produces the pigment melanin (Rajagopal, Kathiravan, & Karthikeyan, 2011). The endemic plant *Cinnamomum malabatrum*'s leaf-associated fungus *C. gloeosporioides* shows antimicrobial activity against human pathogenic bacteria and also in vitro cytotoxicity against the HeLa, MCF-7, and MG63 cancer cell lines (Packiaraj et al., 2016). The fungus produced phenol 3,5-dimethoxy-acetate, 4′-isopropylidene-bis-(2-cyclohexyl) phenol, *N*-didehydrohexacarboxyl-2,4,5-trimethylpiperazine, and 1,2,4-triazolium ylide.

The actinomycetes isolated from *Maytenus aquifolia* and *Putterlickia retrospinosa* have been identified as *Streptomyces setonii* and *Streptomyces sampsonii*, which produce chloropyrrol and chlorinated anthracyclinone with antimicrobial activity (Pullen et al., 2002). Basha, Ogbaghebriel, Yemane, and Zenebe (2012) have isolated the endophytic fungi *Rhizophus oryzae*, *Aspergillus niger*, and *Aspergillus flavus* from *Terminalia brownie.* They showed antimicrobial activity against *S. aureus*, *Enterococcus faecalis*, *P. aeruginosa*, *E. coli*, and *C. albicans. Streptomyces* sp. associated with *Alpinia oxyphylla* is reported to produce 2,6-dimethoxy terephthalic acid (1), yangjinhualine A,

TABLE 1.2 Biological activity of endophytic microbes isolated from medicinal plants.

Medicinal plant	Microbe	Bioactive compounds	Reference
Vitex negundo, Lepisanthes tetraphylla	*Alternaria* sp., *Mycelia sterilia*	Flavonoid	Palanichamy et al. (2018)
Dysosma versipellis	*Fusarium* sp. *Sphaeriothyrium, Penicillium, Arthrinium*	Various metabolites, antioxidant, antibacterial compounds	Tejesvi et al. (2011)
Camptotheca cuminata, Gastrodia elata, and Pinellia ternata	*Alternaria alternate, Phomopsis vaccinii, Fusarium nematophilum. Colletotrichum gloeosporioides, Chaetomium cupreum, Fusarium oxysporum, Armillaria cf. sinapina, Schizophyllum commune, Plectosphaerella cucumerina*	10-Hydroxycamptothecin, camptothecin, ephedrine hydrochloride, gastrodin	Su et al. (2014)
Aloe vera, Mentha arvensis, Ocimum sanctum	*Actinopolyspora* sp., *Micromonospora* sp., *Saccharopolyspora* sp., *Streptomyces* sp.	Hydroxamate, catechol, indole acetic acid	Gangwar et al. (2014)
Azadirachta indica	*Phomopsis*	Melanin	Rajagopal et al. (2011)
Achillea fragrantissima	*Kibdelosporangium* sp., *Kitasatosporia* sp., *Nocardia* sp., *Nocardioides* sp., *Promicromonospora* sp. *Pseudonocardia* sp., *Streptomyces* sp.	Siderophores, chitinase	El-Shatoury et al. (2009)
Cinnamomum malabatrum	*Colletotrichum gloeosporioides*	*N*-Didehydrohexacarboxyl-2,4,5-tri methyl piperazine, 4,4′-isopropylidene-bis-(2-cyclohexyl phenol), Fluoro bis [3-fluorodimethylsilyl-2,2,4,4,6,6-hexamethyl-1,3,5-triaza-2,4,6-risilacyclohexyl]borane, Phenol,3,5-dimethoxy-acetate	Packiaraj et al. (2016)
Maytenu saquifolia, Putterlickia retrospinosa, Putterlickia verrucosa	*Streptomyces setonii, S. sampsonii, Streptomyces* sp.	Celastramycin A and B	Pullen et al. (2002)
Terminalia brownii	*Aspergillus niger* and *A. flavus*	Antimicrobial metabolites	Basha et al. (2012)
Alpinia oxyphylla	*Streptomyces* sp.	2,6-Dimethoxy terephthalic acid	Zhou et al. (2014)
Justicia gendarussa	*Colletotrichum gloeosporioides*	Taxol	Gangadevi and Muthumary (2008)
Miquelia dentata	*Alternaria alternata*	Camptothecin, 9-methoxy camptothecin, 10-hydroxy camptothecin	Shweta et al. (2013)
Phyllanthus niruri, Withania somnifera, Catharanthus roseus, Hemidesmus indicus	*Streptomyces, Nocardioides, Kitasatosporia, Pseudonocardia, Actinomadura, Kibdelosporangium*	Chitinase, cellulases	Priya (2012)
Camellia sinensis	*Alternaria alternata*	Isocoumarin	Wang et al. (2014)
Zingiber officinale, Alpinia galanga	*Streptomyces aureofaciens Streptomyces* sp.	5,7-Dimethoxy-4-*p*-methoxylphenylcoumarin, 5,7-dimethoxy-4-phenylcoumarin actinomycin D	Taechowisan et al. (2003, 2005)
Salvadora persica	*Alternaria* sp.	6,8-Dimethoxy-4-methyl-4H-chromene Benzeneethanol, 1-Octdecene, Cycloeicosane, 1-Butanol, 3-methyl-acetate, 1-Tetradecene, Naphthalene, Phenol, 2,4-di-*t*-butyl-6-nitro	Elgorban et al. (2019)

α-hydroxyacetovanillone, and cyclo(Gly-Trp) (Zhou et al., 2014). Gangadevi and Muthumary (2008) have studied the endophytic fungus *C. gloeosporioides* associated with *Justicia gendarussa* and found it to show cytotoxic activity toward BT 220, H116, Int 407, HL 251, and HLK 210 human cancer cells in vitro. The strain produced taxol which is considered to be a potent anticancer drug. The endophytic bacteria isolated from *Miquelia dentata* (Icacinaceae) synthesizes quinoline alkaloid camptothecin (CPT) which can be used against ovarian and small cell lung cancers (Shweta et al., 2013). Several medicinal plants that are grown in India are associated with endophytic actinomycetes which show antimicrobial activity against *F. solani*, *Macrophomina phaseolina*, *Phytophthora infestans*, and *B. cinerea* (Priya, 2012). The strain *Streptomycetes* sp. produces antifungal compounds and enzymes such as cellulose, chitinase, glucanase, and pectinase.

The endophytic fungus *A. alternata*, from *Camellia sinensis*, has antimicrobial abilities against pathogenic bacteria as well as cytotoxic activities against two human tumor cell lines (Wang, Yang, Wang, Li, & Kong, 2014). The strain produced altenuene derivatives and isocoumarin. *Streptomyces aureofaciens* isolated from the root tissue of *Z. officinale* shows antagonistic activity against *C. musae* and *F. oxysporum.* The strain produces 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin, which both have antimicrobial activity (Taechowisan, Lu, Shen, & Lumyong, 2005).

Elgorban, Bahkali, Farraj, and Abdel-Wahab (2019) have isolated the endophytic fungi *Trichoderma* sp. *Alternaria*, and *Rhizopus arrhizus* from *Salvadora persica* and 62 bioactive chemical compounds have been identified from the ethyl acetate crude extracts of fungal isolates with antimicrobial activity. *S. aureofaciens* synthesized 4-arylcoumarins which have an inhibitory effect on the growth of Lewis lung carcinoma (LLC) in BDF-1 mice (Taechowisan et al., 2005). More detailed studies are needed to increase our knowledge of the role of endophytic fungi in biotechnological processes (Naik, 2019).

1.4 Conclusions

The medicinal plants synthesize various compounds with pharmacological activities, such as antimicrobial, antioxidant, antiulcer, and antihemorrhagic activities. These are used for the treatment of different ailments. The medicinal plant-associated microbes produce various biologically active compounds. Among the antimicrobial compounds, celastramycins, kakadumycins, and javanicin are common metabolites. Several other compounds found in endophytes have antioxidant activities, for example, phenols, tannins, flavonoids, ascorbic acid, carotene, and cajaninstilbene acid. The endophytes associated with medicinal plants that produce biologically active compounds can be considered as potential sources for the discovery of new drugs.

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Chapter 2

Chemodiversity in natural plant populations as a base for biodiversity conservation

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2.1 Biodiversity

The overall variety and variability of life on genetic, population, species, community, and ecosystem levels shape the biological diversity on Earth, along with all interrelations among listed elements and their relations with the environment (Benn, 2010). According to the formal definition given in the international Convention on Biological Diversity (Gaston & Spicer, 2004) "Biological diversity means the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems." Biodiversity is estimated relatively, in a broad time and space range; generally, over the large timescale of 4.5 billion years since life began, it has increased at all levels of organization, while concentrating in smaller areas in regard to spatial distribution, due to complex geological, climatic, historical, and anthropogenic influences (CBD Home, n.d.). Biodiversity is usually discussed at different levels of biological organization, which have hierarchical structure but share the same elements in a dynamic structural−functional complex (Fig. 2.1).

Genetic diversity refers to genome size, number of chromosomes, genes, and nucleotides that distinguish between species, and their variations at population and individual levels. Within-species genetic diversity is expressed by allelic diversity, gene diversity, or nucleotide differences and has a crucial effect on the adaptive ability and fitness of individuals and populations (Hughes, Inouye, Johnson, Underwood, & Vellend, 2008).

Organismal diversity refers to a series of taxonomic categories, including individuals, populations, subspecies and species, genera, families, phyla, kingdoms, and domains. It is often discussed as species diversity and expressed as the number of species (species richness). Numerous indications suggest that population, as a unique set of genetic diversity and environmentally driven adaptations, is accepted as the carrier of within-species diversity. High organismal diversity is based on the number of plant, animal, protist, and fungal species that inhabit the Earth, which number about 10 million. Less than 2 million are known to science (the number could be lower due to the existence of synonyms, homonyms, phenotypes, and cryptic species) (Deans, Yoder, & Balhoff, 2012), and 80% are yet to be described (Mora, Tittensor, Adl, Simpson, & Worm, 2011; Wilson, 2003).

Ecosystem diversity encompasses the broad range of differences among populations, habitats, communities, vegetation ranges, ecosystems, ecoregions, biomes, and biogeographic regions. Unlike organismal diversity, which is supported by taxonomic descriptive data, ecological diversity is more difficult to define because the elements that constitute a community and ecosystem have temporal and spatial dynamics (biodiversity elements) and are inextricably linked to abiotic factors (environmental factors). Biota that coexist in a community establish complex, bidirectional biotic relations, influencing its complexity and maintaining it over a long time continuum (Agrawal et al., 2007). In community structure, various elements belonging to certain functional groups are represented by the species in a community. This concept is a base for functional diversity, that is, the extent of functional differences among the

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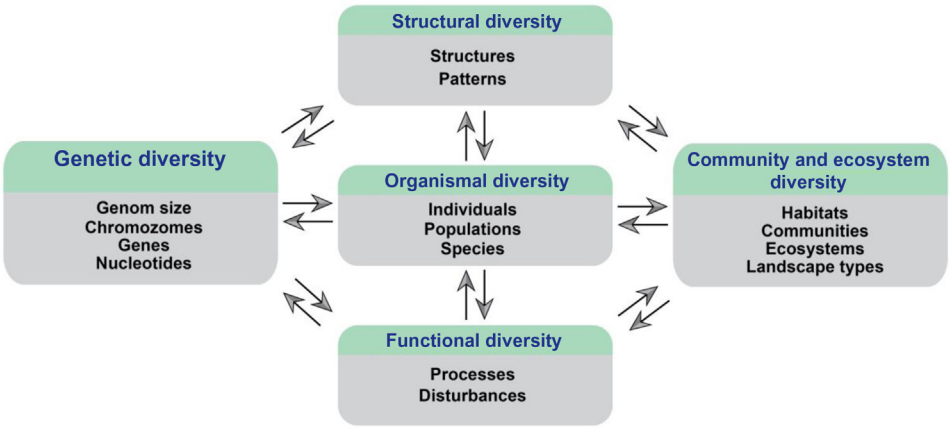

FIGURE 2.1 Levels of biodiversity.

species in a community (Tilman, 2000), based on species' traits (species richness and community composition) (Petchey & Gaston, 2002).

2.2 Biodiversity indicators

Biodiversity cannot be easily quantified due to the complexity of the elements comprising it. It is usually expressed by species richness, that is, number of species. Although a distinct taxonomic category, a species is not clearly defined in some other contexts, primarily genetic and ecological. Genetic and ecological variability within a species in a given habitat determines its functional variability and distribution, which in turn affect the species' role in ecosystem functioning. Therefore each of the mentioned species' attributes should be taken into account in the estimation of biodiversity: taxonomic diversity, abundance, functional traits, distribution, and variability at each level of biological organization (from genes to ecosystem level). Biodiversity also can be considered at different functional levels: as species diversity on a local scale (alpha diversity), the turnover of community composition from place to place or from time to time (beta diversity), and total species diversity in a landscape (gamma diversity, the sum of the previous two) (Whittaker, 1972).

2.3 Biodiversity and biogeography

Distribution of species, communities, and ecosystems along the geographical zones and over geological time is a crucial element in biodiversity. Biogeographic space encompasses a large scale of different latitudes, elevation, habitats, and geomorphology. Among the main attributes that characterize plant species (taxonomic category, life form, distribution, reproduction traits, successional status), geographic range affects within-species diversity the most profoundly (Hamrick & Godt, 1989). In a given geographic range, particular populations of species adapted to specific environmental conditions, which are morphologically clearly distinctive (ecotypes) and/or chemically different (chemotypes), develop.

2.4 Importance of populations for biodiversity

Many biological and related disciplines recognize population as a carrier of species-specific characteristics adapted to habitat requirements and as an important element of biodiversity. As a geographically distinct group of individuals, specimens of the same species, that is, populations, often have a specific genetic structure that contributes to genetic diversity. The phenotypic specificity of populations, based on genes and adaptations to various environmental factors within the distribution range of a species, contributes to organismal diversity. Finally, within each community, populations of different species inhabit specific habitats, respond to abiotic conditions, interact with other species, and participate in ecosystem diversity. It is estimated that, according to the average area of distribution and population density, there may be about 220 distinct populations per eukaryote species (Hughes, Daily, & Ehrlich, 1997), and this number is under consideration because of the destruction of habitats and loss of some populations. With the average number of populations of species (220), there may be more than 2 billion populations over the planet. In the context of biodiversity, a single local species population is a unit of genetic diversity (Tilman, 1996).

2.5 Biodiversity and chemodiversity

The hierarchical sequence of biodiversity from the upper to lower levels moves from ecological biodiversity to organismal diversity and then genetic diversity. Inside a living cell, different types of organelles and subcellular components can be found, and those components below the functional macromolecules are a transitional stage from biological diversity to chemical diversity (Ramesha et al., 2011). Chemical diversity (chemodiversity) encompasses atomic diversity, molecular diversity, macromolecular diversity, chemical diversity spaces, and the diversity of supramolecular assemblages (Testa, Vistoli, Pedretti, & Bojarski, 2009). The application of advanced analytical chemical technologies has enabled the determination of a vast number of chemical components that constitute the chemodiversity within living organisms. An array of chemical components that are synthetized in a living organism shape its metabolome (Oliver, Winson, Kell, & Baganz, 1998). These components can be identified and quantified by sensitive analytical methods, and their number is extremely high (*Escherichia coli* metabolome >2700, human metabolome >40,000).

2.6 Plant chemodiversity

The primary metabolism of plant species relies on a similar biochemical pattern (primary metabolites, that is, amino acids, fatty acids, nucleotides). However, a variety of secondary metabolites among plant taxa is a basis for the inestimable chemodiversity of the plant kingdom. The primary role of secondary metabolites is ecological, in interactions between organisms and between organism and environment. Considering the existence of diversity at all levels of biological organization, the number of possible interactions is very large, and the extensive diversity of metabolites with regard to their chemical structure and properties is not surprising. Many of the secondary metabolites increase the fitness of individuals and populations, and the enzymes included in their biosynthetic pathways are more tolerant to mutations compared to those involved in protein synthesis (Weng, Philippe, & Noel, 2012).

Plant chemical diversity can be expressed on different levels: interindividual chemodiversity, that is, within the plant, among organs or tissues (alpha diversity), chemical compound diversity among plant individuals, such as chemotypes within population (beta diversity) (Moore, Andrew, Külheim, & Foley, 2014), and the total diversity of compounds of all plant populations within a plant community (gamma diversity) (Kessler & Kalske, 2018).

The pronounced natural plant chemodiversity can be explained by several facts: secondary metabolites originate from precursors from primary metabolism, which are chemically simple molecules, and the number of their possible combinations is large; biosynthesis of secondary metabolites is driven by enzymes of a similar type, usually encoded by multiple genes; some biosynthetic enzymes can produce multiple products from the same precursor; great functional divergence of biosynthetic enzymes may be explained by their low substrate specificity, and result in modifications of secondary metabolism; and, interindividual diversity in secondary metabolism, expressed as temporal, spatial, and organ-related variability (Kessler & Kalske, 2018).

2.7 Chemodiversity—analytical approaches

Plant chemodiversity is assessed by the comprehensive examination of secondary metabolites by different qualitative and quantitative chemical analyses. The estimated number of metabolites in the plant kingdom is over 200,000 (Weckwerth, 2003), and vary in a large range of concentrations and chemical properties. Depending on the analytical technique, a wide spectrum of metabolites can be detected and identified using the appropriate databases. Traditional targeted chemical analyses applied to a specific compound or group of compounds identify only a part of secondary metabolism diversity. This analytical approach revealed the existence of different chemotypes within distinct plant populations. In contrast, the metabolomics approach (screening of all metabolites) gives results that are more difficult to interpret because of the great diversity between samples depending on multiple factors (plant developmental stage, diurnal rhythm, differences between plant organs and tissues) (Macel, Van Dam, & Keurentjes, 2010). Metabolomics is a powerful tool in determining a plant's biochemical phenotype, containing a complex of small molecule metabolites with a mass <1000 Da in the assumed number of tens of thousands (Hall et al., 2002). This method is promising not only for identifying biologically active compounds, but also their precursors that became the basis for subsequent modification (Bykov, 2016).

2.8 Bioprospecting

Biodiversity, and the underlying chemodiversity, is the main base for bioprospecting, that is, searching for new species that can serve as sources for medicinal compounds and other biologically sourced compounds of economic

importance (Banerjee, 2017). Multidisciplinary approaches encompassing biological, chemical, pharmaceutical, and biomedical disciplines are applied for the purpose of bioprospecting, and the overall biodiversity, including bacteria, fungi, plants, invertebrates, and vertebrates, is the subject of numerous studies (Bishop et al., 2015; Carvalho et al., 2016; Indraningrat, Smidt, & Sipkema, 2016; Leal, Puga, Serôdio, Gomes, & Calado, 2012; Ooi et al., 2016).

2.9 Plant biodiversity and biomedicine

Biodiversity is a foundation of human health and well-being, being a basic structure of ecosystem functioning and the provision of goods and services. The links between biodiversity, environmental health, and human health are evident in various spatial and temporal scales (Romanelli et al., 2015). Climate change, disaster risk, food and water security, infectious diseases, traditional medicine, mental health, and biomedical/pharmaceutical progress are dependent on the maintenance of biodiversity. Due to its exceptional role in the survival and health of humans, biodiversity maintenance and conservation are one of the highest goals in the present decade (Benn, 2010).

Pharmacology has been founded on biodiversity from ancient times. Over 50% of pharmaceutical compounds rely on biological resources, and about 80% of the human population use natural medicines for healthcare. Among the various biological sources for biomedical investigation and the production of effective and safe drugs, biologically active substances of plant origin amount to 60%–65% (Bykov, 2016). The procedure of inserting plant biodiversity into biomedicine is multistepped and long, with several phases: acquisition (determining the plant source, extract preparation, preliminary bioassay), discovery (basic screening, confirmatory screening, chemical isolation and identification), preclinical development (provision of a sufficient amount of raw/synthetized material, preliminary animal studies, provision of large-scale supply, advanced animal studies, investigation of new drugs according to standard procedures), clinical development (clinical trials—assessment of safety in healthy volunteers, clinical trials—safety and efficacy in patients, clinical trials—efficacy in a larger number of patients, in comparison with current treatments, application of new drug, commercialization, postmarket surveillance) (Chivian, 2002).

2.10 Targeted plant studies in the discovery of potential new drug candidates

The existence of within-taxa diversity of ecotypes, phenotypes, and chemotypes is of great importance to biomedicine. Numerous plant species are intensively investigated for the purpose of chemical profiling and searching for specific chemical components that singly or synergistically exhibit biological activity.

At the same time, many biomedical studies do not take into consideration population biodiversity, disregarding the determination of plant phenotype/chemotype prior to biological activity testing. Also, in published papers there are many omissions in the taxonomic determination or specification of a taxon, which can be misleading in the interpretation of results (Popović, Matić, Bojović, Stefanović, & Vidaković, 2016; Rzhetsky, Seringhaus, & Gerstein, 2008). Very few biomedical studies consider population or the individual diversity of biological material, which is an additional challenge to the repeatability of bioassays. The discovery of new drugs from natural plant populations is crucial for healthcare, disease prevention, and wellness (Cordell, 2000; Neergheen-Bhujun et al., 2017; Scannell & Bosley, 2016). However, for the successful identification of biologically active compounds, the selection of plant taxa is crucial, and many investigators propose a nonrandom approach in taxa selection that is based on biodiversity, chemodiversity, and ecology (Schwikkard & Mulholland, 2014).

2.11 Balkan Peninsula—geographic and biological diversity

The Balkan Peninsula is the southeasternmost peninsula of Europe, bordered by the Black Sea, Sea of Marmara, Aegean Sea, Mediterranean Sea, Ionian Sea, and Adriatic Sea. The Danube, Sava, and Kupa rivers are commonly considered to be the northern border between the Balkan Peninsula and central Europe. Due to the geographic, historical, and political features of the region, its northern boundary line is usually extended to include the territories of several countries: Slovenia, Croatia, Serbia, Bosnia and Herzegovina, Montenegro, Albania, Macedonia, continental Greece (including Peloponnesus), Bulgaria, European Turkey, and southeast Romania (Fig. 2.2).

The region is characterized by mountain ranges in a NW to SE direction, a long coastal zone, and the most diverse types of natural landscapes found on the continent. The wide range of climatic zones that exist within the peninsula are due to its location on the border between temperate and subtropical climates, as well as complex oceanic and continental influences (Nojarov, 2017). Profound diversity, geological, geographical, climatic, and

ecological conditions over a long period of time have brought about the formation of a wide array of regions, habitats, and community relations for the wildlife. There are several zonal vegetation units on the Balkan Peninsula: maritime woodlands and maquis, semideserts, steppes, different types of forests, high-mountain rocks and tundra, and wet high-mountain grasslands, mountain rocks, and arid grasslands (Horvat, Glavač, & Ellenberg, 1974). There is a large number of biomes (approximately 250) and each of them contains roughly 35 biotopes, which implies a great diversity of biotopes and vast richness of flora and fauna (Savić, 2008). The Balkan Peninsula is considered a world hotspot and center of biodiversity (Stevanović, Vasić, & Radović, 1996). The mountains of the Balkan Peninsula are listed among the 156 centers of vegetal biodiversity in the world, and are one of the six in Europe (Davis, Heywood, & Hamilton, 1994).

2.12 Research on plant population chemodiversity in the Balkans

Plant biodiversity is crucial for ecosystem stability and has been investigated from different aspects. In this regard, chemical diversity among plant species and populations plays an important role in biodiversity maintenance and, consequently, ecosystem functioning: the establishment of species richness in plant community composition, numerous ecological interactions between community members, plant/soil interactions, responses to global ecological disturbances, and climate changes. Recent studies in the field of population chemodiversity have provided a lot of information relevant to chemotaxonomy, abiotic and biotic influences on secondary metabolism, the formation of a chemical compound database, and the targeting of particular sites/populations as rich natural sources of potentially pharmacologically important compounds. The summary of studies on population chemistry on the territory of the Balkan Peninsula presented in this paper has included all papers published on this topic collected by searching peer-reviewed scientific literature using internet search engines with access to all the major databases in the life sciences (Scopus, Web of Science, Science Direct, Wiley InterScience, Google Scholar, Thompson Reuters, and Springer Link). The following keywords were used in our search of Titles, Abstracts, and Keywords in published literature: "population," "phytochemistry," "chemistry," "secondary metabolites," "essential oils," "phenolics," "Balkan," and the names of each of the countries located on the Balkan Peninsula. After collecting a database of 119 articles, further analysis was conducted by mapping the locations of investigated populations, their distances, analytic procedures (plant part, analytical material), chemical compounds, and determined differences among obtained data.

In total, 120 taxa were investigated—species, subspecies, and varieties (Table 2.1)—and almost all studies confirmed differences (qualitative, quantitative, or both) in chemical composition among the studied populations. The minimal number of populations within the taxa was 2, and the maximal was 38; the smallest distance between two studied populations was 4 km, and the largest was 1160 km. The majority of studies focused on the total chemoprofiling of plant essential oils (all components), or on the determination of terpene components of the oils. Both approaches were the basis for distinguishing the chemotypes within the taxa. Another analytical approach was the extraction with solvents of different polarity, and the obtained extract was used to determine phenolics, flavonoids, alkaloids, fatty acids, fatty alcohols, hydrocarbons, aliphatic esters, furanocoumarins, iridoid glycosides, and organic acids. Out of the total number of studied genera (40), the largest number of populations comprised studies of the following genera: *Achillea*, *Juniperus*, *Pinus*, *Satureja*, *Salvia*, and *Thymus*; these studies also covered large research areas (Fig. 2.3).

2.13 Biomedical importance of population chemodiversity research

In the presented review, several of the main types of analytical approach were applied: total chemoprofiling of a plant's essential oil (and/or focus on the dominant component of essential oils—terpenes), the determination of total phenolic/flavonoid/anthocyanin content, and targeted quantification of selected secondary metabolites. From the available database, selected compounds with confirmed biomedical significance are presented in Table 2.2.

The use of plant essential oils in human healthcare is a part of traditional medicine with increasing potential exploitation in clinical medicine. Although there are still many issues regarding their incorporation in clinical practice (i.e., standardization and quality control, human clinical and experimental pharmacology and toxicology, safety, biomedical applications, and others), there is undeniable evidence of their benefits to human health. Both botanical origin and chemical uniqueness (chemotype) are important because the same species name may be used to label products with different chemical compositions. In traditional medicine, plant essential oils are widely used for treating respiratory, digestive, gynecological, andrological, endocrine, cardiovascular, neural, and dermatological diseases (Firenzuoli et al., 2014). Their beneficial effects are usually based on the joint activity (synergistic, additive, antagonistic) of an oil's

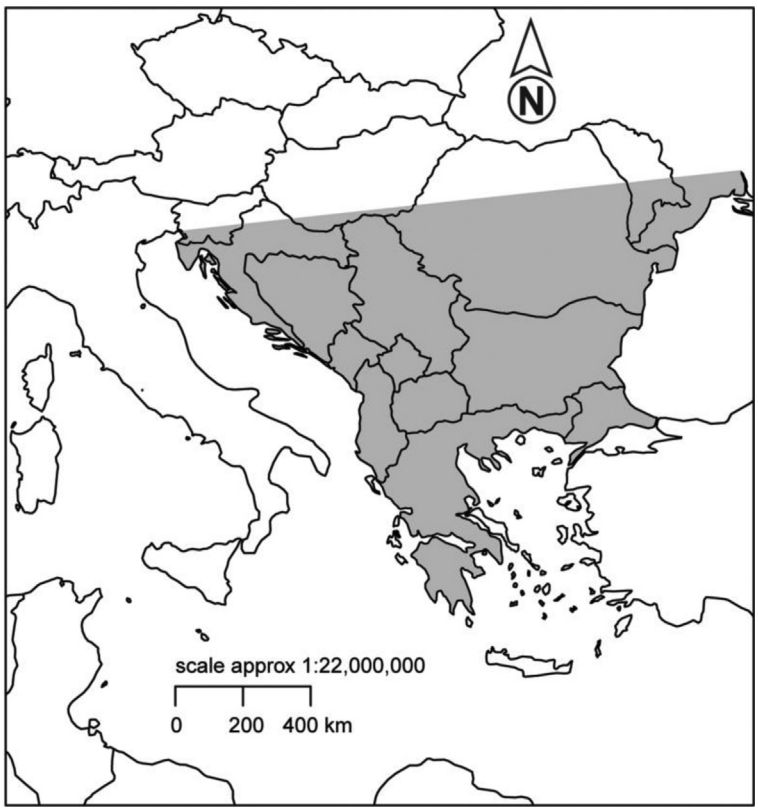

FIGURE 2.2 Balkan Peninsula, southeast Europe.

chemical components. However, its individual constituents can show toxic, allergic, and carcinogenic effects, or are contraindicative.

The essential oils of some species included in this study are widely used in aromatherapy and for dermatological issues (*Thymus vulgare*, *Origanum vulgare*), and certain constituents of the essential oils listed in the table also have proven biomedical potential (Hazzit, Baaliouamer, Faleiro, & Miguel, 2006). Out of the total number of different components of essential oils found in investigated taxa, we selected the most frequent compounds and found that 11 were reported as bioactive agents, 7 in different model systems (1,8-cineole, chamazulene, α-pinene, spathulenol, L-menthone, carvone, camphene), and 4 were studied in humans (carvacrol, linalool, D-limonene, thymol). The large group of phenolic compounds analyzed in a given dataset was divided into several smaller groups: flavonoids, anthocyanins, diarylheptanoids, phenolic and chlorogenic acids, and other phenolics. Flavonoids are secondary metabolites widely distributed in the form of glycosides in plants, with a pronounced bioactive role as anticancer, antibacterial, antioxidative, and antiinflammatory agents (Panche, Diwan, & Chandra, 2016). In the investigated species, the following flavonoids with pronounced biomedical activity were determined: hispidulin, catechin, epicatechin, chrysoeriol, apigenin, quercetin, and anthocyanins. Diarylheptanoids are a small class of secondary plant metabolites with a wide variety of biological activities (Alberti, Riethmüller, & Béni, 2018), and some of the compounds detected in the listed species have proved their potential for further investigations (oregonin, hirsutanonol, platyphylloside). Ferulic acid, *p*-hydroxycinnamic acid, gallic acid, and chlorogenic acid are powerful antioxidants with both a protective role against oxidative-damage diseases and health-promoting effects (Ghasemzadeh & Ghasemzadeh, 2011). Other phenolics (imanin, hypericin, verbascoside) and one phloroglucinol derivative (hyperforin) listed in Table 2.2 have also shown important biological activities. The long history of the use of alkaloids in medicine has resulted in many of these compounds being incorporated in pharmaceutical products as a basis for synthetic and semisynthetic drugs (Babbar, 2015). In this review, we detected several alkaloids with biomedical importance (glaucine, isocorydine, lycorine, haemanthamine, hordenine, tazettine, and narciclasine), and one was tested in humans (galanthamine). Furanocoumarins have been reported as toxic agents against bacteria, fungi, viruses, and insects, and their antipathogenic function was confirmed in several biomedical studies (pimpinellin, isopimpinellin, bergapten, isobergapten, sphondin, xanthotoxin, and imperatorin). Polyacetylenes are also known as antimicrobial agents, and falcarinol, detected in the listed species, has displayed six different biological activities.

TABLE 2.1 Population chemodiversity of investigated Balkan taxa with relevant data on analytical procedure (plant parts, analytical material), general class of secondary metabolites, number and distance of populations, between-population variability based on quantitative (Qt) and qualitative (Ql) differences in analyzed compounds, selected compounds, and literature cited.

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Achillea abrotanoides (Vis.) Vis.	AP	EO	Terpenoids	2	~5.3	Qt + Ql +	α-Thujone, 1,8-cineole, *trans*-sabinene hydrate (Hanlidou, Kokkini, & Kokkalou, 1992)
Achillea ageratifolia (Sibth. & Sm.) Benth. & Hook.f	L	EE	Phenolics	10		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol, kaempferol glycosides (Franzén, 1988)
Achillea ageratifolia subsp. *aizoon* (Griseb.) Heimerl	L	EE	Phenolics	4		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol, kaempferol glycosides (Franzén, 1988)
Achillea ageratifolia subsp. *serbica* (Nyman) Heimerl	L	EE	Phenolics	4		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea ambrosiaca (Boiss. & Heldr.) Boiss.	L	EE	Phenolics	4		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea clavennae L.	L	EE	Phenolics	/		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea fraasii Sch. Bip.	L	EE	Phenolics	/		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea millefolium L.	AP	EO	Terpenoids	3	max 50	Qt + Ql +	Chamazulene, β-caryophyllene, β-pinene, lavandulyl acetate, *trans*-chrysanthenyl acetate, germacrene D (Dajic Stevanovic et al., 2015)
	L	EO	Terpenoids	4		Qt + Ql +	1,8-Cineol, camphor, ascaridole, fragnanol (Karagiannidis, Panou-Filotheou, Lazari, Ipsilantis, & Karagiannidou, 2010)
Achillea pindicola subsp. *integrifolia* (Halácsy) Franzén	L	EE	Phenolics	5		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea pindicola Hausskn.	L	EE	Phenolics	3		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Achillea umbellata Sibth. & Sm.	L	EE	Phenolics	12		Ql +	3,6-Di-*O*-methyl-kaempferol, hispidulin, nepetin, cirsiliol (Franzén, 1988)
Alnus glutinosa (L.) Gaertn.	B	EE	Phenolics	2	18	Qt + Ql - +	Hirsutanonol-5-*O*-β-D-glucopyranoside, oregonin, hirsutanonol, alnuside A, platyphylloside, rubranoside A (Vidaković et al., 2018)
Alnus incana (L.) Moench	B	EE	Phenolics	2	11	Qt + Ql - +	Hirsutanonol-5-*O*-β-D-glucopyranoside, oregonin, hirsutanonol, alnuside A, platyphylloside, rubranoside A (Vidaković et al., 2018)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Anthemis auriculata Boiss.	/	EO	Terpenoids	2	~100	Qt + Ql +	Spathulenol, β-caryophyllene, β-eudesmol (Saroglou, Dorizas, Kypriotakis, & Skaltsa, 2006)
Anthemis chia L.	/	EO	Terpenoids	4	~180	Qt + Ql +	*cis*-Chrysanthenyl acetate, β-caryophyllene, germacrene D (Saroglou et al., 2006)
Anthemis tomentosa Boiss.	/	EO	Terpenoids	2	~105	Qt + Ql +	*cis*-Chrysanthenyl acetate, β-caryophyllene, germacrene D (Saroglou et al., 2006)
Arbutus unedo L.	L	EE	Phenolics Polyphenols	2	/	Qt +	Total phenolics, total flavonoids (Pavlović, Branković, Kovačević, Kitić, & Veljković, 2011)
Artemisia alba Turra	AP	EO	Terpenoids	2	~200	Qt + Ql +	Spathulenol, artemisia ketone, camphor, 1,8-cineole (Radulović & Blagojević, 2010)
Ballota macedonica Vandas	AP	EO	Terpenoids	2	~80	Qt + Ql +	Carotol, germacrene D, β-caryophyllene (Đorđević, Jovanović, Zlatković, & Stojanović, 2016)
Calamintha menthifolia Host	AP	EO	Terpenoids	2	~10.8	Qt + Ql +	Piperitone oxide (Hanlidou, Kokkini, Bosabalidis, & Bessière, 1991)
	AP	EO	Terpenoids	7	/	Qt + Ql +	Piperitenone oxide, pulegone, menthone, isomenthone (Karousou, Hanlidou, & Lazari, 2012b)
Calamintha nepeta (L.) Savi	L	EO	Terpenoids	2	~10.8	Qt + Ql +	Pulegone, menthone (Karagiannidis et al., 2010)
	AP	EO	Terpenoids	7	/	Qt + Ql +	Piperitenone oxide, pulegone, menthone, isomenthone (Karousou et al., 2012b)
Chrysanthemum coronarium L.	Fl	EO	Terpenoids	2	~115	Qt + Ql +	*trans*-Chrysanthenyl acetate, *trans*-chrysanthenyl isovalerate, *cis*-chrysanthenyl acetate, camphor (Basta, Pavlović, Couladis, & Tzakou, 2007)
Cornus mas L.	Fr	ME	Phenolics	2	142	Qt +	Neochlorogenic acid, quercitrin, isoquercetin, hyperoside, rutoside, querciturone (Bajić-Ljubičić, Popović, Matić, & Bojović, 2018; Popović, Matić, Bajić-Ljubičić, Tešević, & Bojović, 2018)
Cornus sanguinea L.	Fr	ME	Phenolics	2	142	Qt +	Neochlorogenic acid, quercitrin, isoquercetin, hyperoside, rutoside, querciturone (Popović et al., 2018; Popović, Bajić-Ljubičić, Matić, & Bojović, 2017)

Cotinus coggygria Scop.	AP	EO	Terpenoids	3	~125	Qt + Ql +	Limonene, α-pinene, terpinolene, myrcene, sabinene, terpin-4-ol (Tzakou, Bazos, & Yannitsaros, 2005)
Eryngium campestre L.	AP	EE	Phenolics	3	73	Qt +	/ (Nebija et al., 2009)
Galanthus elwesii Hook.f.	AP, Fl	EE	Alkaloids	25	/	Qt + Ql +	Hordenine-, homolycorine-, lycorine-, galanthamine-type alkaloids (Berkov, Bastida, Sidjimova, Viladomat, & Codina, 2011)
	AP, Fl	H_2SO_4 extract	Alkaloids		/	Qt + Ql +	Haemanthamine, crinine, galanthamine-type alkaloids (Berkov, Sidjimova, Evstatieva, & Popov, 2004)
Galanthus nivalis L.	AP, Fl	EE	Alkaloids	7	/	Qt + Ql +	Narciclasine-, galanthamine-, lycorine-, haemanthamine-, tazettine-type alkaloids (Berkov et al., 2011)
Glaucium flavum Crantz	L	ME	Fatty acids Fatty alcohols Phenolics Alkaloids	5	~172	Qt + Ql +	Alkaloid fraction: glaucine, isocorydine; lipid fraction: octadecane, hexadecanol, hexadecanoic acid, octadecatrienoic acid; phenolic fraction: ferulic acid, *p*-hydroxycinnamic acid (Nikolova, Berkov, Doycheva, Stoyanov, & Stanilova, 2018)
Heracleum pyrenaicum subsp. *pollinianum* (Bertol.) F. Pedrotti & S. Pignatti	Rh, R, Fr	EO	Terpenoids Aliphatic esters Furanocoumarins	3	43	Qt + Ql +	β-Pinene, terpinolene, octyl acetate, pimpinellin, isopimpinellin, sphondin, bergapten, isobergapten (Ušjak, Drobac, Niketić, & Petrović, 2018)
Heracleum sphondylium subsp. *sibiricum* (L.) Simonk.	Rh, R, Fr	EO	Terpenoids Phenylpropenes Fatty acid esters and alcohols Furanocoumarins	4	max 110	Qt + Ql +	Limonene, α-pinene, β-pinene, methyl eugenol, elemicin, octyl acetate, *n*-octanol, pimpinellin, isopimpinellin, sphondin, bergapten, isobergapten (Ušjak et al., 2018)
Heracleum sphondylium subsp. *verticillatum* (Pančić) Brummitt	Rh, R, Fr	EO	Terpenoids Aliphatic esters Furanocoumarins	2	~4	Qt + Ql (+)	β-Pinene, limonene, intermedeol, octyl acetate, octyl isobutanoate, octyl 2-methylbutanoate, pimpinellin, isopimpinellin, sphondin, bergapten, isobergapten (Ušjak et al., 2018)
Hypericum perfoliatum L.	AP	EO	Terpenoids	2	~200	Qt + Ql +	α-Pinene, *n*-nonane, δ-cadinene, β-pinene, γ-cadinene (Couladis, Baziou, Petrakis, & Harvala, 2001)
Hypericum perforatum L.	L	NaOH, HCl extract	Phenolics	2	50	Qt +	Hyperforin, hypericin, imanin (Nikolic & Zlatkovic, 2010)
	AP	EO	Terpenoids Hydrocarbons Fatty alcohols	5	max 75	Qt + Ql (+)	2-Methyl-octane, α-pinene, β-caryophyllene, caryophyllene oxide, *n*-tetradecanol (Hajdari et al., 2014)
	AP	EO	Terpenoids	11	max 162	Qt + Ql +	α-Pinene, β-pinene, β-caryophyllene, β-selinene, caryophyllene oxide (Bardhi, Stefkov, Karapandzova, Cvetkovikj, & Kulevanova, 2015)
	AP	ME	Phenolics	5	max 200	Qt +	Catechin, epicatechin, quercetin, neochlorogenic acid, cyanidin-3-*O*-glucoside, hypericin, hyperforin (Sarrou et al., 2018)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Hyssopus officinalis subsp. *aristatus* (Godr.) Nyman	AP	EO	Terpenoids Phenolics	5	max 130	Qt + Ql +	1,8-Cineol, *cis*-pinocamphone, β-pinene, *trans*-pinocamphone, chlorogenic acids (Hajdari et al., 2018)
Juniperus communis L.	N	HE	Terpenoids	10	max 624	Qt +	α-Pinene, β-myrcene, sabinene, limonene (Hajdari et al., 2015a)
	N	EO	Terpenoids	8	max 790	Qt + Ql +	α-Pinene, sabinene, γ-terpinene (Radoukova et al., 2018)
	T	EO	Terpenoids	3	max 100	Qt + Ql +	α-Pinene, sabinene (Sela et al., 2013)
Juniperus communis var. *saxatilis* Pall.	N	HE	Hydrocarbons	10	max 624	Qt + Ql (+)	*n*-Tritriacontane, *n*-nonacosane, *n*-hentriacontane, *n*-pentatriacontane (Rajčević, Janaćković, Dodoš, Tešević, & Marin, 2014)
	N	EO	Terpenoids	2	~360	Qt + Ql (+)	α-Pinene, sabinene, δ-3-carene, limonene (Radoukova et al., 2018)
Juniperus deltoides R.P. Adams	T	EO	Terpenoids	4	max 542	Qt + Ql (+)	α-Pinene, limonene (Rajčević, Janaćković, Bojović, Tešević, & Marin, 2013)
	T	EO	Terpenoids	10	max 558	Qt + Ql +	α-Pinene, limonene (Rajčević, Janaćković, Dodoš, Tešević, & Marin, 2015)
	N	HE	Hydrocarbons	9	max 717	Qt + Ql (+)	*n*-Tritriacontane (Rajčević et al., 2014)
Juniperus excelsa M. Bieb.	L	EO	Terpenoids	2	~160	Qt + Ql +	α-Pinene, sabinene (Sela, Karapandzova, Stefkov, Cvetkovikj, & Kulevanova, 2015)
Juniperus oxycedrus L.	Fr	EO	Terpenoids	5	max 80	Qt + Ql (+)	β-Myrcene, α-pinene, limonene, germacrene D (Hajdari et al., 2014)
Juniperus phoenicea var. *turbinata* (Guss.) Parl.	T	EO	Terpenoids	7	max 923	Qt +	α-Pinene, β-phellandrene, germacrene D (Rajčević, Labus, Dodoš, Novaković, & Marin, 2018)
Juniperus phoenicea L.	L, Fr	EO	Terpenoids	2	/	Qt + Ql +	α-Pinene, myrcene, δ-3-carene (Vourlioti-Arapi, Michaelakis, Evergetis, Koliopoulos, & Haroutounian, 2012)
Lamium album L.	/	ME	Iridoid glycosides	2	~20	Qt (+)	Lamalbid, sesamoside, lamiol, 5-deoxylamiol, shanzhiside methyl ester, caryoptoside, penstemoside, lamioside, barlerin (Alipieva, Kokubun, Taskova, Evstatieva, & Handjieva, 2007)

Lamium garganicum L.	/	ME	Iridoid glycosides	3		Qt (+)	Lamalbid, sesamoside, lamiol, 5-deoxylamiol, shanzhiside methyl ester, caryoptoside, penstemoside, lamioside, barlerin (Alipieva et al., 2007)
Lamium amplexicaule L.	/	ME	Iridoid glycosides	2	/	Qt (+)	Lamalbid, sesamoside, lamiol, 5-deoxylamiol, shanzhiside methyl ester, caryoptoside, penstemoside, lamioside, barlerin (Alipieva et al., 2007)
Lamium maculatum (L.) L.	/	ME	Iridoid glycosides	2	/	Qt (+)	Lamalbid, sesamoside, lamiol, 5-deoxylamiol, shanzhiside methyl ester, caryoptoside, penstemoside, lamioside, barlerin (Alipieva et al., 2007)
Lamium purpureum L.	/	ME	Iridoid glycosides	4	/	Qt (+)	Lamalbid, sesamoside, lamiol, 5-deoxylamiol, shanzhiside methyl ester, caryoptoside, penstemoside, lamioside, barlerin (Alipieva et al., 2007)
Leucojum aestivum L.	AP, Fl, Bl	ME	Alkaloids	31	/	Qt + Ql +	Galanthamine, homolycorine, lycorine, crinine (Berkov et al., 2013)
	Bl	ME	Alkaloids	18	/	Qt + Ql +	Galanthamine, epinorgalanthamine, narwedine, lycorine, ungiminorine (Georgieva et al., 2007)
Marrubium peregrinum L.	AP, Fl	EO	Terpenoids	2	~71	Qt + Ql (+)	(*Z*)-β-Farnesene, (*E*)-β-Farnesene (Lazari, Skaltsa, & Constantinidis, 1999)
Melissa officinalis L.	AP, Fl	EO	Terpenoids	3	/	Qt + Ql (+)	β-Pinene, sabinene, caryophyllene oxide, β-caryophyllene (Basta, Tzakou, & Couladis, 2005)
Melissa officinalis subsp. *altissima* (Sm.) Arcang.	L	EO	Terpenoids	5	/	Qt + Ql +	Geranial, carvacrol, neral, pulegone, sabinene (Karagiannidis et al., 2010)
Mentha pulegium L.	AP, Fl	EO	Terpenoids	38	/	Qt + Ql +	Pulegone, piperitone, menthone, isomenthone, piperitenone, isopiperitenone (Kokkini, Hanlidou, Karousou, & Lanaras, 2004)
	AP, Fl	EO	Terpenoids	2	/	Qt + Ql +	Piperitone, isomenthone (Kimbaris et al., 2017)
Mentha longifolia (L.) L.	AP	EO	Terpenoids	2	/	Qt + Ql +	Piperitenone oxide, 1,8-cineole, *trans*-piperitone epoxide, carvone, limonene (Koliopoulos, Pitarokili, Kioulos, Michaelakis, & Tzakou, 2010)
Mentha spicata L.	/	EO	Terpenoids	2	/	Qt + Ql +	Piperitenone, linalool, piperitenone oxide (Kofidis, Kokkini, & Bosabalidis, 2011)
Micromeria croatica (Pers.) Schott	AP	EO	Terpenoids	3	max 60	Qt + Ql +	Caryophyllene oxide, β-caryophyllene (Kremer, Stabentheiner, Dunkić, et al., 2012)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Micromeria dalmatica Benth.	AP	EO	Terpenoids	13	/	Qt +	β-Pinene, limonene, germacrene D, menthone, isomenthone, pulegone, piperitone, piperitenone, piperitenone oxide (Karousou, Hanlidou, & Lazari, 2012a)
						Ql +	
Micromeria graeca (L.) Benth. ex Rchb.	AP, Fl	EO	Terpenoids	2	~20	Qt +	Caryophyllene oxide, *epi*-α-bisabolol, linalool, β-chamigrene (Tzakou & Couladis, 2001)
						Ql +	
Micromeria juliana (L.) Benth. ex Rchb.	L	EO	Terpenoids	2	/	/	Linalool, linalyl acetate, carvacrol, thymol (Karagiannidis et al., 2010)
Micromeria longipedunculata Bräuchler	AP	EO	Terpenoids	4	max 85	Qt +	Spathulenol, piperitone oxide, piperitone (Kremer et al., 2014)
						Ql (+)	
Nepeta argolica Bory & Chaub.	AP, Fl	EO	Terpenoids	2	/	Ql +	1,8-Cineole, δ-cadinene, nepetalactones (Skaltsa, Lazari, Loukis, & Constantinidis, 2000)
Nepeta spruneri Boiss.	AP. Fl	EO	Terpenoids	6	/	Qt +	Caryophyllene oxide, myrtenol, 1,8-cineole, nepetalactone (Hanlidou, Karousou, & Lazari, 2012)
						Ql +	
Origanum vulgare L.	AP	EO	Terpenoids	2	/	Qt +	Carvacrol, caryophyllene oxide (Hodaj-Çeliku et al., 2017)
	AP	EO	Terpenoids	11	max 1000	Qt +	Sabinene, β-caryophyllene, germacrene D, caryophyllene oxide (Lukas, Schmiderer, & Novak, 2015)
						Ql +	
	AP	EO	Terpenoids	6	max 450	Qt +	Sabinene, β-caryophyllene, germacrene D, caryophyllene oxide (Lukas et al., 2015)
						Ql +	
	AP	EO	Terpenoids	3	max 140	Qt +	Thymol, carvacrol (Lukas et al., 2015)
Origanum vulgare subsp. *hirtum* (Link) Ietsw.	AP, Fl	EO	Terpenoids	23	/	Qt +	Carvacrol, thymol, γ-terpinene, *p*-cymene (Vokou, Kokkini, & Bessiere, 1993)
						Ql (+)	
	AP	EO		30	/	Qt +	/ (Gavalas, Kalburtji, Kokkini, Mamolos, & Veresoglou, 2011)
	L	EO	Terpenoids	2	/	/	*trans*-Sabinene hydrate, terpin-4-ol, terpinene (Karagiannidis et al., 2010)
Ornithogalum umbellatum L.	AP	EE	Phenolics	4	max 140	Qt +	Chlorogenic acid, gallic acid, coumaric acid, *p*-hydroxibenzoic, quercetin, rutin, epicatechin (Rat et al., 2016)
						Ql +	
Picea omorika (Pancic) Purk.	T, N	EO	Terpenoids	4	max 74	Qt +	Bornyl acetate, camphene, α-pinene (Nikolić, Tešević, Đorđević, Marin, & Bojović, 2009)
						Ql (+)	
	N	HE	Hydrocarbons Fatty alcohols	3	max 64	Qt +	*n*-Heptacosane, *n*-nonacosane, *n*-hentriacontane, nonacosan-10-ol (Nikolić et al., 2013)

Pinus heldreichii Christ	T, N	EO	Terpenoids	4	max 150	Qt +	/ (Nikolić, Ristić, Tešević, Marin, & Bojović, 2011)
	N	HE	Hydrocarbons Fatty alcohols	4	max 150	Qt +	Nonacosan-10-ol, *n*-tricosane, *n*-heptacosane, *n*-pentacosane (Nikolić et al., 2012a)
	T, N	EO	Terpenoids	2	140	/	Germacrene D, limonene, α-pinene (Nikolić et al., 2015)
	T, N	EO	Terpenoids	4	max 150	Qt + Ql (+)	Limonene, α-pinene, germacrene D, β-caryophyllene (Nikolić, Ristić, Bojović, & Marin,2007)
Pinus heldreichii var. *pančići* Fukarek	N	HE	Hydrocarbons	7	max 41	Qt (+)	*n*-Heptacosane, *n*-tricosane, *n*-pentacosane, *n*-nonacosane (Nikolić et al., 2010)
Pinus mugo Turra	T, N	EO	Terpenoids	4	max 34	Qt +	δ-3-carene, β-phellandrene, α-pinene, β-myrcene (Bojović et al., 2016)
	T, N, C	EO	Terpenoids	6	max 90	Qt (+)	α-iPnene, β-pinene, δ − 3-carene, limonene, β-phellandrene, β-caryophyllene, germacrene D (Hajdari et al., 2015b)
Pinus nigra J.F. Arnold	T, N	EO	Terpenoids	7	max 300	Qt +	α-Pinene, germacrene D (Šarac et al., 2013)
Pinus nigra var. *banatica* Georgescu & Ionescu	N	HE	Hydrocarbons	4	max 212	Qt +	*n*-Pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-tricosane (Mitić et al., 2018)
	N	HE	Hydrocarbons	4	max 212	Qt (+)	*n*-Heptacosane, *n*-pentacosane, *n*-tricosane, *n*-nonacosane (Mitić, Zlatković, Jovanović, Stojanović, & Marin, 2016)
Pinus nigra subsp. *dalmatica* (Vis.) Franco	N	HE	Hydrocarbons Terpenoids	2	31	Qt +	*n*-Pentacosane, *n*-heptacosane, *n*-tricosane, *n*-nonacosane, pimaradiene, sandaracopimaradiene (Mitić et al., 2018)
Pinus nigra var. *nigra*[a]	N	HE	Hydrocarbons	3	max 10	Qt +	*n*-Tricosane, *n*-pentacosane, *n*-heptacosane (Bojović et al., 2012)
	N	HE	Hydrocarbons	3	max 132	Qt +	*n*-Pentacosane, *n*-heptacosane, *n*-tricosane, *n*-nonacosane (Mitić et al., 2018)
	N	HE	Hydrocarbons	2	max 123	Qt (+)	*n*-Heptacosane, *n*-pentacosane, *n*-tricosane, *n*-nonacosane (Mitić et al., 2016)
Pinus nigra subsp. *pallasiana* (Lamb.) Holmboe	N	HE	Hydrocarbons Fatty alcohols	6	max 260	Qt +	*n*-Pentacosane, *n*-heptacosane, *n*-tricosane, *n*-nonacosane, *cis* − 9-octadecen-1-ol (Mitić et al., 2018)
	N	HE	Hydrocarbons Fatty alcohols	3	max 138	Qt +	*n*-Heptacosane, *n*-pentacosane, *n*-tricosane, *n*-nonacosane, *cis* − 9-octadecen-1-ol (Mitić et al., 2016)
Pinus nigra var. *gocensis* Georgev.	N	HE	Hydrocarbons	2	88	Qt +	*n*-Tricosane, *n*-pentacosane, *n*-heptacosane, *n*-docosane, *n*-tetracosane, *n*-triacontane, *n*-dotriacontane (Bojović et al., 2012)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Pinus peuce Griseb.	T, N	EO	Terpenoids	3	max 51	Qt +	α-Pinene, germacrene D (Nikolić, Ristić, Bojović, & Marin, 2008)
	T, N	EO	Terpenoids	3	max 51		/ (Nikolić et al., 2011)
	T, N	EO	Terpenoids	2	132	Qt +	α-Pinene, germacrene D, β-pinene, camphene (Nikolić et al., 2014)
	N	EO	Terpenoids	7	max 97	Qt +	α-Pinene, β-phellandrene, β-pinene (Hajdari et al., 2016a)
						Ql (+)	
	N	HE	Hydrocarbons Fatty alcohols	3	max 51	Qt +	Nonacosan-10-ol, *n*-nonacosane, *n*-pentacosane, *n*-heptacosane, *n*-tricosane (Nikolić et al., 2012b)
	T, N	EO	Terpenoids	3	max 150	Qt +	α-Pinene, β-pinene, germacrene D (Karapandzova et al., 2014)
Pistacia terebinthus L.	L, Fr, G	EO	Terpenoids	2	23	Qt +	α-Pinene, limonene, β-ocimene, β-pinene, sabinene, (*Z*)-β-ocimene (Pulaj, Mustafa, Nelson, Quave, & Hajdari, 2016)
						Ql +	
Pulicaria dysenterica (L.) Gaertn.	AP	EO	Terpenoids	2	~190	Qt +	(*Z*)-Nerolidol, caryophyllene oxide, (*E*)-nerolidol, β-caryophyllene (Basta, Tzakou, Couladis, & Pavlović, 2007)
						Ql (+)	
Rosmarinus officinalis L.	/	EO	Terpenoids	10	max 993	Qt +	1,8-Cineole, camphor, α-pinene, borneol, myrcene, camphene (Lakušić, Ristić, Slavkovska, Šinžar-Sekulić, & Lakušić, 2012)
						Ql (+)	
Salvia fruticosa Mill.	L	EO	Terpenoids	3	max 45	Qt (+)	1,8-Cineole, camphor (Schmiderer, Torres-Londoño, & Novak, 2013)
Salvia officinalis L.	L, Fl	EO	Terpenoids	11	max 123	Qt +	α-Thujone, 1,8-cineol, camphor, α-humulene, viridiflorol, manool (Couladis, Tzakou, Mimica-Dukić, Jančić, & Stojanović, 2002)
	L	EO	Terpenoids	12	max 123	Qt +	1,8-Cineole, *cis*-thujone, *trans*-thujone, camphor, viridiflorol (Stešević et al., 2014)
	AP	EO	Terpenoids	25	max 440	Qt +	*cis*-Thujone, camphor, *trans*-thujone, 1,8-cineole, β-pinene (Jug-Dujaković et al., 2012)
	L	EO	Terpenoids	23	max 285	Qt +	1,8-Cineole, α-thujone, β-thujone, camphor, β-caryophyllene, α-humulene, viridiflorol (Schmiderer et al., 2013)
	AP	EO	Phenolics	2	~230	–	/ (Duletić-Laušević, Alimpić, Pavlović, Marin, & Lakušić, 2016)
	L	EO	Terpenoids	25	max 1160	Qt +	β-Pinene, 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor, α-humulene, viridiflorol, manool (Cvetkovikj, Stefkov, Karapandzova, Kulevanova, & Satović, 2015)
	AP	EO	Terpenoids	2	max 1160	Qt +	Camphor, α-thujone (Hodaj-Çeliku et al., 2017)

Salvia sclarea L.	AP	EO	Terpenoids	2	~42	Qt + Ql (+)	Linalyl acetate, linalool, geranyl acetate, α-terpineol (Pitarokili, Couladis, Petsikos-Panayotarou, & Tzakou, 2002)
	L	EO	Terpenoids	2	/	/	/ (Karagiannidis et al., 2010)
Salvia tomentosa Mill.	AP	EO	Terpenoids	19	/	Qt + Ql +	α-Pinene, β-pinene, camphor, camphene, 1,8-cineole, *cis*-thujone, borneol (Hanlidou, Karousou, & Lazari, 2014)
Salvia verticillata L.	L, Fl	EO	Terpenoids Hydrocarbons	3	max 170	Qt + Ql +	Germacrene D, β-caryophyllene (Krstic, Malencic, & Anackov, 2006)
Sambucus nigra L.	L, Fl, Fr	ME	Phenolics	8	max 110	Qt +	Hydroxycinnamic acid derivatives, isorhamnetin glycosides, kaempferol glycosides, quercetin glycosides, flavanols, flavanones, anthocyanins (Senica, Stampar, Veberic, & Mikulic-Petkovsek, 2017)
Satureja horvatii Šilić	AP	EO	Terpenoids	2	300	Qt + Ql +	Thymol (Lakušić, Ristić, Slavkovska, Milenković, & Lakušić, 2011)
Satureja horvatii subsp. *macrophylla* (Halácsy) Baden	AP, Fl	EO	Terpenoids	36	/	Qt + Ql (+)	Carvacrol, thymol, *p*-cymene, linalool (Dardioti, Hanlidou, Lanaras, & Kokkini, 2010)
Satureja montana L.	AP	EO	Terpenoids Phenolics	7	max 154	Qt +	Myrcene, *p*-cymene, γ-terpinene, linalool, thymol, carvacrol, viridiflorol (Hajdari et al., 2016b)
	AP	EO	Terpenoids Phenolics	7	max 440	Qt +	Carvacrol, thymol (Dunkić et al., 2012)
	L	HE	Hydrocarbons	4	max 323	Qt +	*n*-Nonacosane, *n*-hentriacontane (Dodoš et al., 2015)
Satureja montana subsp. *montana*[a]	AP	EO	Terpenoids	2	60	Qt +	*p*-Cymene, linalool, borneol (Slavkovska, Jancic, Bojovic, Milosavljevic, & Djokovic, 2001)
	L	HE	Hydrocarbons	4	max 323	Qt +	*n*-Nonacosane, *n*-hentriacotane (Dodoš et al., 2015)
Satureja montana subsp. *pisidia* (Wettst.) Šilić	L	HE	Hydrocarbons	2	72	Qt +	*n*-Nonacosane, *n*-hentriacontane (Dodoš et al., 2015)
Satureja montana subsp. *variegata* (Host) P.W. Ball	L	HE	Hydrocarbons	2	47	Qt (+)	*n*-Nonacosane, *n*-hentriacontane (Dodoš et al., 2015)
Satureja pilosa Velen.	AP, Fl	EO	Terpenoids	19	/	Qt +	γ-Terpinene, *p*-cymene, linalool, borneol, thymol, carvacrol (Dardioti, Karousou, Lanaras, & Kokkini, 2012)
Satureja subspicata Bartl. ex Vis.	AP	EO	Terpenoids Phenolics Polyphenols	7	max 440	Qt +	α-Eudesmol, β-eudesmol, spathulenol (Dunkić et al., 2012)
Satureja subspicata subsp. *liburnica* Šilić	L	HE	Hydrocarbons	5	max 155	Qt +	*n*-Nonacosane, *n*-hentriacontane (Dodoš, Rajčević, Tešević, & Marin, 2017)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Satureja subspicata subsp. *subspicata*[a]	L	HE	Hydrocarbons	4	max 158	Qt +	*n*-Nonacosane, *n*-hentriacontane (Dodoš et al., 2017)
Seseli rigidum Waldst. & Kit.	AP, R, Fr	EO	Terpenoids Fatty alcohols	7	max 167	Qt +	Falcarinol, α-pinene, limonene, β-phellandrene, sabinene, germacrene B, carotol, germacrene D, β-sesquiphellandrene (Marčetić, Kovačević, Lakušić, & Lakušić, 2017)
	AP, R, Fr	EO	Terpenoids Fatty alcohols	7	max 167	Qt +	Falcarinol, methyl linoleate, α-muurolene (Marčetić, Lakušić, Lakušić, & Kovačević, 2013)
Sideritis clandestina subsp. *peloponnesiaca* (Boiss. & Heldr.) Baden	AP, Fl	EO	Terpenoids	2	/	Qt + Ql +	α-Pinene (Koutsaviti, Bazos, Milenković, Pavlović-Drobac, & Tzakou, 2013)
Sideritis raeseri Boiss. & Heldr.	AP	EO	Terpenoids	7	max 250	Qt + Ql +	β-Pinene, α-pinene, α-copaene, sabinene, limonene (Qazimi, Stefkov, Karapandzova, Cvetkovikj, & Kulevanova, 2014)
	AP	HE EE	Hydrocarbons Phenolics	5	max 140	Qt - +	Hentriacontane, nonacosane, heptacosane (Karapandzova et al., 2013)
	AP	ME	Phenolics	12	max 260	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, isoscutellarein glycosides, hypolaetin glycoside, methylhypolaetin glycosides (Petreska Stanoeva et al., 2015)
	aerial parts	ME	Phenolics	7	max 150	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, hypolaetin glycoside, apigenin 7-(4″-*p*-coumaroylglucoside), methylisoscutellarein glycoside (Petreska et al., 2011)
Sideritis scardica Griseb.	Fl	EO	Terpenoids	6	max 130	Qt +	α-Pinene, β-pinene, β-bisabolene, benzyl benzoate, *m*-camphorene (Trendafilova, Todorova, Evstatieva, & Antonova, 2013)
	AP	EO	Terpenoids Fatty alcohols	5	max 90	Qt +	β-Caryophyllene, β-pinene, α-pinene, 1-octen-3-ol (Qazimi et al., 2014)
	AP	HE, EE	Hydrocarbons Phenolics	9	max 90	Qt - +	Hentriacontane, nonacosane, heptacosane (Karapandzova et al., 2013)
	AP	EE	Phenolics	11	max 330	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, isoscutellarein glycosides, hypolaetin glycoside, methylhypolaetin glycosides (Petreska Stanoeva et al., 2015)
	AP	ME	Phenolics	9	max 240	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, hypolaetin glycoside, apigenin 7-(4″-*p*-coumaroylglucoside), methylisoscutellarein glycoside (Petreska et al., 2011)
	AP	EO	Terpenoids Fatty alcohols	3	max 310	Qt + Ql +	α-Cadinol, octadecenol (Kostadinova et al., 2007)

Sideritis syriaca L.	AP	ME	Phenolics	2	25	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, isoscutellarein glycosides, hypolaetin glycoside, methylhypolaetin glycosides (Petreska Stanoeva et al., 2015)
Sideritis taurica Steph. ex Willd.	AP	ME	Phenolics	3	max 1160	Qt + Ql +	Neochlorogenic acid, lavandulifolioside, verbascoside, isoscutellarein glycosides, hypolaetin glycoside, methylhypolaetin glycosides (Petreska Stanoeva et al., 2015)
	AP	EO	Terpenoids Fatty aldehydes	2	~50	Qt +	β-Caryophyllene, germacrene D, caryophyllene oxide, (*E*)-nerolidol, *n*-nonanal, linalool (Kukić et al., 2006)
Stachys germanica subsp. *heldreichii* (Boiss.) Hayek	/	EO	Terpenoids	2	~98	Qt + Ql +	Germacrene D, β-caryophyllene, caryophyllene oxide (Skaltsa, Demetzos, Lazari, & Sokovic, 2003)
Stachys officinalis (L.) Trevis.	L, R	ME		3	max 43	Qt - +	/ (Hajdari, Mustafa, Franz, & Novak, 2010)
	L, Fl, R	EO	Terpenoids Hydrocarbons Fatty alcohols	3	max 43	Qt + Ql(+)	α-Pinene, 1-octen-3-ol, β-bourbonene, β-caryophyllene, germacrene D, α-pinene, *trans*-β-farnesene, nonane (Hajdari, Mustafa, Franz, & Novak, 2011)
Stachys spruneri Boiss.	/	EO	Terpenoids	4	~22	Qt + Ql (+)	β-Caryophyllene, δ-cadinene (Skaltsa, Mavrommati, & Constantinidis, 2001)
Stachys swainsonii subsp. *argolica* (Boiss.) Nyman	/	EO	Terpenoids	3	~25	Qt + Ql (+)	β-Caryophyllene, δ-cadinene (Skaltsa et al., 2001)
	AP, Fl	SE	Phenolics	3	~25	Ql +	Apigenin, chrysoeriol, xanthomicrol, salvigenin, eriodictyol, eupatorin, cosmoside, luteolin glucoside, chrysoeriol glucoside, chrysoeriol 7-*O*-(3″-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside (Skaltsa et al., 2007)
Stachys swainsonii subsp. *melangavica*[a]	AP, Fl	SE	Phenolics	3	~27	Qt + Ql (+)	Apigenin, chrysoeriol, xanthomicrol, salvigenin, eriodictyol, eupatorin, cosmoside, luteolin glucoside, chrysoeriol glucoside, chrysoeriol 7-*O*-(3″-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside (Skaltsa et al., 2007)
Stachys swainsonii subsp. *scyronica* (Boiss.) Pithos & Damboldt	/	EO	Terpenoids	2	~7	Qt + Ql (+)	β-Caryophyllene, δ-cadinene (Skaltsa et al., 2001)
	AP, Fl	SE	Phenolics	2	~7	Ql +	Apigenin, chrysoeriol, xanthomicrol, salvigenin, eriodictyol, eupatorin, cosmoside, luteolin glucoside, chrysoeriol glucoside, chrysoeriol 7-*O*-(3″-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside (Skaltsa et al., 2007)
Stachys swainsonii subsp. *swainsonii* (accept. *Stachys swainsonii Benth.*)	/	EO	Terpenoids	3	~24	Qt + Ql (+)	β-Caryophyllene, δ-cadinene (Skaltsa et al., 2001)
	AP, Fl	SE	Phenolics	3	~24	Ql +	Apigenin, chrysoeriol, xanthomicrol, salvigenin, eriodictyol, eupatorin, cosmoside, luteolin glucoside, chrysoeriol glucoside, chrysoeriol 7-*O*-(3″-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside (Skaltsa et al., 2007)
Stachys sylvatica L.	L, Fl	EO	Terpenoids Phenolics	3	max 60	Qt +	α-Pinene, β-pinene, germacrene D (Hajdari, Novak, Mustafa, & Franz, 2012)

(*Continued*)

TABLE 2.1 (Continued)

Taxon	Plant part	An. mat.	General class of SM	Pop no.	Dist (km)	Pop diff.	Selected compounds
Taxus baccata L.	T, N	EO	Terpenoids Fatty alcohols	3	max 206	Qt +	1-Octen-3-ol, (3*Z*)-hexen-1-ol, myrtenol (Stefanović et al., 2016)
Teucrium arduinii L.	AP	EO	Terpenoids	11	max 496	Qt + Ql (+)	β-Caryophyllene, germacrene D, caryophyllene oxide, pulegone, piperitone oxide (Kremer et al., 2015)
	AP	ME	Phenolics polyphenols	6	max 450	Qt - +	Kremer, Stabentheiner, Jurišić Grubešić et al. (2012)
	AP	ME	Phenolics	6	max 450	Qt + Ql +	Phenolic acids, chlorogenic acid (Šamec et al., 2015)
Teucrium chamaedrys L.	AP	ME	Phenolics	6	∼235	Qt +	Rutin (Zdraveva, Pavlova, Krasteva, & Pencheva, 2018)
Thymus atticus Celak.	AP	EO	Terpenoids	3	∼103	Qt + Ql +	(*E*)-Nerolidol, β-caryophyllene, germacrene D, myrcene, 1,8-cineole (Tzakou & Constantinidis, 2005)
Thymus glabrescens Benth.	AP	EO	Terpenoids	5	max 93	Qt +	Thymol, γ-terpinene, p-cymene (Dajić-Stevanović, Šoštarić, Marin, Stojanović, & Ristić, 2008)
Thymus leucospermus Hartvig	AP, Fl	EO	Terpenoids	3	/	Qt + Ql (+)	*p*-Cymene, γ-terpinene, thymol (Pitarokili, Constantinidis, Saitanis, & Tzakou, 2014)
Thymus longicaulis C. Presl	AP, S	EO	Terpenoids	2	∼132	Qt + Ql +	Carvacrol, geraniol, thymol (Chorianopoulos et al., 2004)
Thymus pannonicus (accept. *Thymus pulegioides* subsp. *pannonicus* (All.) Kerguélen)	L	EO	Terpenoids	3	max 175	Qt + Ql +	Neral, geranial, α-pinene, germacrene D (Sostaric, Arsenijevic, Acic, & Stevanovic, 2012)
Thymus parnassicus Halácsy	AP	EO	Terpenoids	3	∼58 km	Qt (+) Ql +	β-Caryophyllene, linalyl acetate, γ-terpinene (Tzakou & Constantinidis, 2005)
Thymus teucrioides subsp. *candilicus* (Beauverd) Hartvig	AP, Fl	EO	Terpenoids	6	/	Qt + Ql +	*p*-Cymene, borneol, β-caryophyllene, γ-terpinene, thymol (Pitarokili et al., 2014)
Thymus teucrioides subsp. *teucrioides*	AP, Fl	EO	Terpenoids	4	/	Qt + Ql (+)	*p*-Cymene, borneol, β-caryophyllene (Pitarokili et al., 2014)
Vaccinium myrtillus L.	Fr	ME	Phenolics Organic acids	6	max 50	Qt +	Cyanidin, delphinidin, malvidin, petunidin and peonidin glycosides, citric acid, quinic acid (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2015)

[a]*Full botanical name not available. Selected compounds are either dominantly present in analytical material, or taxon-specific compounds.* AP, *Aerial parts;* L, *leaves;* B, *bark;* Fl, *flowers;* Fr, *fruits;* R, *root;* Rh, *rhizome;* N, *needles;* T, *twigs;* Bl, *bulbs;* C, *cones;* G, *galls;* S, *seeds;* EO, *essential oil;* ME, *methanol extract;* EE, *ethanol extract;* HE, *hexane extract;* CE, *chloroform extract;* SE, *extraction with series of solvents with different polarity;* Qt, *quantitative analysis;* Ql, *qualitative analysis;* + , *significant differences;* − , *nonsignificant differences;* (), *small differences;* - + , *differences found partially, for some compounds.*

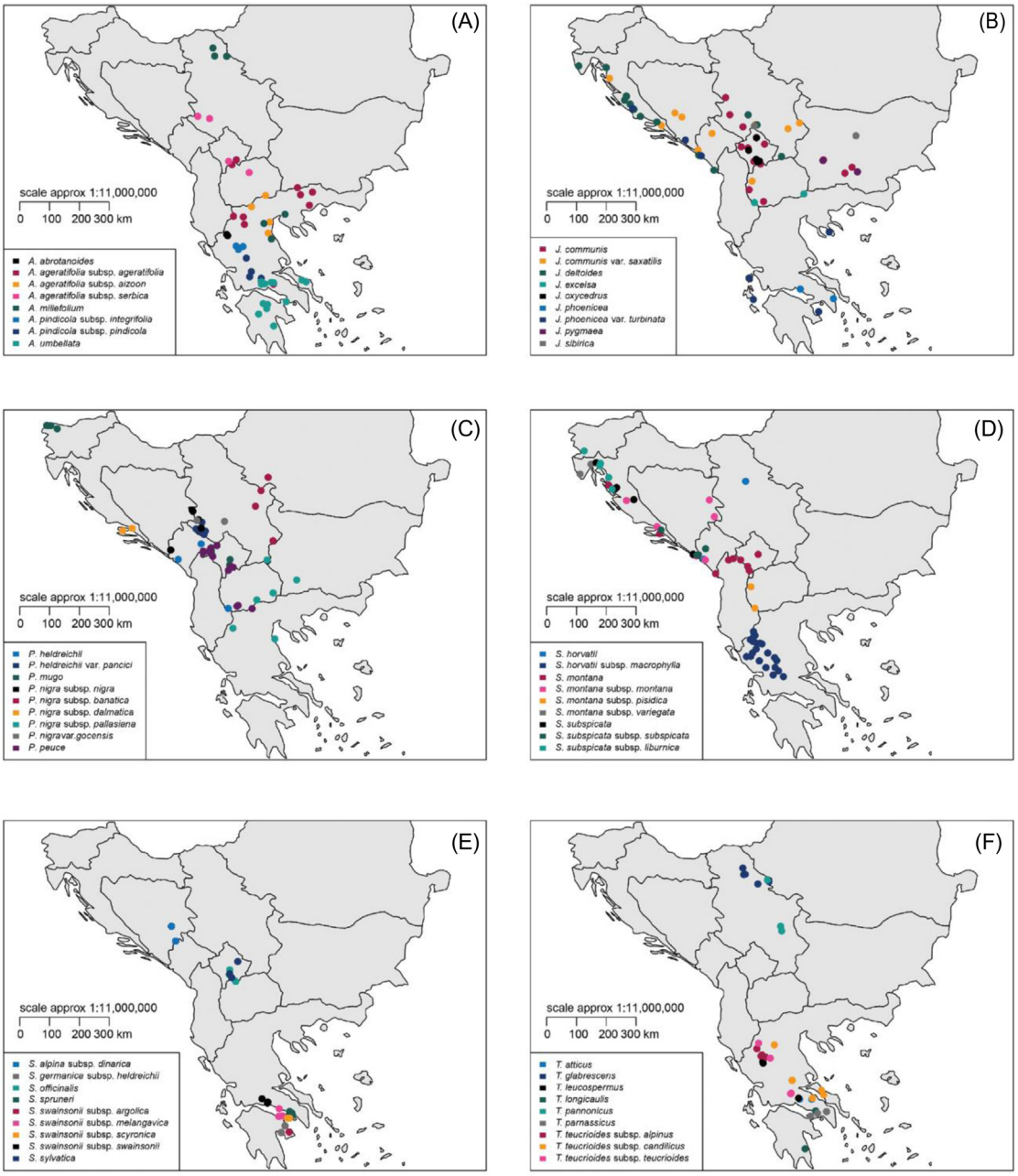

FIGURE 2.3 Locations of the populations of the most investigated genera in Balkan Peninsula (1) *Achillea*, (2) *Juniperus*, (3) *Pinus*, (4) *Satureja*, (5) *Salvia*, and (6) *Thymus*.

2.14 Chemodiversity as a base for biodiversity conservation

Biodiversity maintenance and conservation are very important goals of humankind. The growing interest in biomedicine in biodiversity arises from the interest in chemodiversity that is at its core. Chemodiversity is closely linked to genetic and environmental diversity. On the other hand, genetic and environmental diversity contribute to the overall functional diversity within the plant taxa, and consequently modulate an array of community interactions and ecosystem functioning. It has been shown that chemical diversity within plant taxa leads to decreased herbivory and diseases in plant populations (Hughes et al., 2008) and affects community diversity through trophic relations (Glassmire et al., 2016; Richards et al., 2015). The functional traits of the biota coexisting in an ecosystem are crucial for the understanding of established relationships and the strategic planning of protection and conservation of natural resources. It is estimated by the

TABLE 2.2 Selected secondary metabolites with biomedical importance.

		Biomedical significance		
Class of secondary metabolites	**Selected compounds**	**Studies in vitro, in vivo, animal models**	**Studies in humans**	**References**
Flavonoids	Hispidulin	Anticonvulsant activity Cytotoxicity Neuroprotective activity		Kavvadias et al. (2004) Han et al. (2018) Huang, Huang, and Ning (2018)
	Catechin, epicatechin	Protection against oxidative DNA damage		Delgado, Haza, García, and Morales (2009)
	Chrysoeriol	Antidiabetic activity Antihyperlipidemic activity		Baskaran, Viswanathan Pugalendi, and Saravanan (2015)
	Apigenin	Antitumor activity Antiangiogenic activity		Fang et al. (2005)
	Quercetin	Antioxidant activity Antitumor activity Antiinflammatory activity Chemopreventive activity		Jeong, An, Kwon, Rhee, and Lee (2009)
	Anthocyanins	Anticancer activities		Parisi et al. (2014)
Diarylheptanoids	Oregonin	Antioxidative activity Antiinflammatory activity Anticancer activity Immunosuppressive activity Cytotoxicity		Choi et al. (2010) and Choi et al. (2008)
	Hirsutanonol, platyphylloside	Cytotoxicity		Choi et al. (2008)
Phenolic and chlorogenic acids	Ferulic acid	Antioxidant activity Antiinflammatory activity Chemopreventive activity Hepatoprotective activity Antithrombotic activity Beneficial effects on human sperm mobility and viability Potential chemopreventive effect Against alzheimer's disease		Yan et al. (2001)
	p-Hydroxycinnamic acid	Antihyperglycemic activity Antihyperlipidemic activity Antioxidant activity		Ambika, Saravanan, and Thirumavalavan (2013)

	Gallic acid	Antioxidant activity Anti-allergic activity Antiinflammatory activity Anti-mutagenic activity Anti-carcinogenic activity		Cirillo et al. (2011)
	Chlorogenic acid	Antioxidant activity Improves lipid and glucose metabolism		Li, Chang, Ma, and Yu (2009)
Other phenolics	Imanin		Antibiotic	Nikolic and Zlatkovic (2010)
	Hypericin	Antiviral activity Antineoplastic activity Photocytotoxicity in cancer cells		Agostinis et al. (2000)
	Verbascoside	Antioxidant activity Antiinflammatory activity		Cardinali et al. (2013)
		Potential therapeutic value for the treatment of cognition deficits		Filho et al. (2012)
Phloroglucinol derivative	Hyperforin	Antidepressant activitiy Anxiolytic activity Antibacterial activity Cognition-enhancing activiy Antioxidant activity Anticarcinogenic activity		Zanoli (2004)
Alkaloids	Galanthamine		Treatment in Alzheimer's disease, myasthenia gravis and muscular dystrophy, residual poliomyelitis paralysis symptoms, trigeminal neuralgia and other forms of neuritides	Heinrich and Lee Teoh (2004)
	Glaucine	Antiinflammatory activity Bronchodilator		Cortijo et al. (1999)
	Isocorydine	Anticancer activity Antiviral activity Antiparasitic activity		Xin, Zhang, Zhang, Di, and Liu (2018)
	Lycorine	Antitumor activity Antiangiogenic activity Antiviral activity Antibacterial activity Antimalarial activity Antiparasite activity Antioxidant activity Hepatoprotective activity Antiinflammatory activity		Khalifa, Attia, Fahim, and Kamel (2018)
	Haemanthamine	Antitumor activity Antileukemic activity		Hroch et al. (2016)

(*Continued*)

TABLE 2.2 (Continued)

		Biomedical significance		
Class of secondary metabolites	**Selected compounds**	**Studies in vitro, in vivo, animal models**	**Studies in humans**	**References**
		Antioxidant activity Antiviral activity Anticonvulsant activity Antimalarial activity		
	Hordenine	Antidiabetic activity Antiinflammatory activity Antifibrotic activity Antibacterial activity Antibiotic activity		Su et al. (2018)
	Tazettine	Antimalarial activity Antineoplastic activity		Acosta, Pigni, Oleas, and Bastida (2014)
	Narciclasine	Anticancer activity Antiviral activity Bacteriostatic activity Antifungal activity Antiparasitic activity		Kornienko and Evidente (2008)
furanocoumarins	Pimpinellin Isopimpinellin Bergapten Isobergapten Sphondin	Antimycobacterial activity		Walasek, Grzegorczyk, Malm, and Skalicka-Woźniak (2015)
	Sphondin Bergapten Isobergapten Xanthotoxin	Antitumor activity		Sumiyoshi, Sakanaka, Taniguchi, Baba, and Kimura (2014)
	Pimpinellin Bergapten Sphondin	Antimycobacterium tuberculosis activity		Sharghi et al. (2017)
	Imperatorin	Antiseizure activity Vasodilation activity Antineoplastic activity Antimicrobial activity		Liu, Cao, and Wang (2010)
Polyacetilenes	Falcarinol	Antifungal activity Antiinflammatory activity Antiplatelet-aggregatory activity Antibacterial activity Cytotoxic activity Anticancer activity		Christensen and Brandt (2006)

Monoterpenes Sesquiterpenes Diterpenes	1,8-Cineole	Antiinflammatory activity Vascular activity Intestinal smooth muscle relaxant activity Bronchodilatatory activity Nasal decongestant activity Antitussive activity		Nascimento et al. (2009)
	Chamazulene	Antioxidant activity Antiinflammatory activity		Safayhi, Sabieraj, Sailer, and Ammon (1994)
	α-Pinene	Antiinflammatory activity Antioxidant activity Antinociceptive activity Antiulcerogenic activity Antibacterial activity Antifungal activity Antileichmania activity Anticancer activity		Bouzenna, Hfaiedh, Giroux-Metges, Elfeki, and Talarmin (2017)
	Spathulenol	Antifibrotic activity		Lou et al. (2019)
	Carvacrol		Antimicrobial activity Antioxidant activity Anticancer activity	Sharifi-Rad et al. (2018)
	Linalool		Antioxidative activity Reduces blood pressure and pulse rate in patients with carpal tunnel syndrome (CTS)	Seol, Kang, Lee, and Seol (2016)
	L-Menthone	Cooling activity Pain relief activity Antidepressant activity		Xue et al. (2015)
	D-Limonene		Gallstone dissolution activity Heartburn relief activity Anticancer activity	Sun (2007)
	Carvone	Antimicrobial activity Nematicidal activity Antitumor activity Antioxidant activity Antihyperglycemic activity		Muruganathan and Srinivasan (2016)
	Thymol		Antitussive activity	Gavliakova et al. (2013)
	Camphene	Antioxidant activity Antiinflammatory activity Antimicrobial activity Antihepatosteatotic activity		Kim, Choi, Choi, Choi, and Park (2014)

WWF (World Wide Fund for Nature) that over two-thirds of the 50,000 medicinal plants in use today are harvested from the wild, of which 4000−10,000 may now be endangered (Hamilton, 2003). Defining existing chemotypes within natural populations would contribute to the sustainable usage of natural resources and create stronger links between biodiversity and human health (Alves & Rosa, 2007; McNeely, 2006).

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Chapter 3

Harnessing the potential of plant biodiversity in health and medicine: opportunities and challenges

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3.1 Introduction

The renewed interest in plants as natural sources for drugs makes it timely to discuss the ongoing challenges and opportunities for the future of drug discovery. Eleven percent of drugs listed as essential medicines by the World Health Organization (WHO) are exclusively derived from plants (Veeresham, 2012). Although the growing challenge associated with plants as a drug discovery source is obtaining access to the material, there are still technical challenges associated with identification, compound yield, seasonal variation in chemical composition, how to create a sustainable supply, ownership, legislation, and patentability (Atanasov et al., 2015). These challenges must be overcome to increase the study of the remaining 400,000 species of vascular plants; especially in an era where most wild plants are threatened with extinction (Botanic Gardens Conservation International, 2019). Plant-derived natural compounds still top the list of successful modern therapeutics (e.g., vincristine, camptothecin, taxol, quinine derivatives, artemisinin, morphine, codeine digitoxin, atropine, tubocurarine, etc.). In addition, approximately 2000 new plant species are discovered annually, enlarging the pool for drug bioprospecting. Given the vast plant biodiversity that remains to be explored, there is tremendous opportunity for identifying new compounds from plants for medicine.

Because each plant species contains thousands to millions of different, potentially useful molecules, one can imagine the economic and health prosperity that could be achieved by exploring more of our planet's biodiversity. While new research technologies have emerged to streamline screening of molecules and complex mixtures from diverse biological sources, the loss of biodiversity is accelerating, reducing the potential for discovery of new natural compounds with therapeutic properties (Cao & Kingston, 2009; Kingston, 2011; Newman & Cragg, 2016). Nevertheless, the increasing morbidity and mortality from both communicable and noncommunicable diseases, along with drug resistance (World Health Organization, 2019), call for imperative action to prevent the loss of potential drugs due to biodiversity extinction (Cao & Kingston, 2009). The continued loss of biodiversity threatens compound discovery and benefits that could be used to protect communities and the biodiversity they depend on. Large-scale environmental genomics and metabolomics provide an unprecedented opportunity for renewed efforts in protecting biodiversity, indigenous knowledge, and personalized medicine. This raises an urgent need to facilitate legal and practical frameworks that promote and regulate cataloging, characterizing, and at the same time, protecting biodiversity. For example, new models are needed for harnessing biodiversity for biomedical applications that merge with policy development for sustainable harvesting.

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To create new paradigms for conservation based on the ecosystem service of drug discovery, partnerships are needed among scientists and pharmaceutical industries to identify species and isolate compounds, social scientists to assess community needs and ensure community empowerment, and policy makers to produce legislation to guide protection and to develop models for sustainable agriculture and research involving modern biotechnology and chemistry. Given the potential for overexploitation and the unknown amount of compounds lost, this scenario of biodiversity—a focus on drugs lost from species extinction—is largely unexplored.

3.2 Medicinal plants—a historical perspective to contemporary uses

The use of natural products to mitigate human ailments is deeply rooted in antiquity (Jaspars et al., 2016; Wright, 2019). The knowledge and use of medicinal plants by different ancient civilizations is supported by texts, including the Chinese book "Pen T'Sao," describing some 350 medicinal plants in China (2500 BCE), the Vedas in India (2000 BCE), and the Elder Papyrus in Egypt (1550 BCE) amongst others (Fowler, 2006; Petrovska, 2012). In view of safeguarding the medicinal knowledge, many authors have attempted to compile a list of medicinal plants known to them, in the form of books to be consulted by future generations. For instance, Pliny the Elder (CE 23−79) wrote "Historia Naturalis," John Mesue (CE 850) wrote "De Re Medica" and Ibn Baitar (CE 1197−1248) compiled the "Liber Magnae Collectionis Simplicum Alimentorum Et Medicamentorum" describing 1000 plants with medicinal value (Petrovska, 2012). Over the millennia, plants have provided the architectural blueprints for sophisticated ethnomedicinal practices in different regions of the world. Many of these herbal medicinal systems are still being practised today.

Even though modern healthcare is characterized by the availability of commercially synthesized molecules, a vast majority of the world's population continues to rely on plants as their primary source of healthcare. Estimates from the WHO show that in Africa up to 80% of the population still relies on traditional medicines, including plant-derived medicines (WHO, 2002). The second *States of the World's Plants report* compiled by experts at the Royal Botanic Gardens Kew, listed 28,187 plant taxa that are currently documented with medicinal uses in different regions around the globe. The trend of medicinal plant use is predominant in China and India, followed by Colombia and countries in southern Africa (Chen et al., 2016). An estimated 11,146 and 7500 medicinal plant taxa representing 41% and 44% of the native flora are reported in China and India, respectively (Hamilton, 2004). The high usage rate of medicinal plants in developing countries is attributed to the fact that both the plants and their associated traditional knowledge are more easily accessible and affordable to the general population (Willis, 2017), as can be seen from the high ratios of traditional medicine practitioners compared to medical doctors (Rukangira, 2002). In addition, the global demand and trade for medicinal plants is expanding (Mafimisebi, Oguntade, Ajibefun, Mafimisebi, & Ikuemonisan, 2013).

3.3 Current status of higher plants in drug discovery

In addition to providing herbal galenicals for disease mitigation, medicinal plants contributed to 25% of life-saving pharmaceutical drugs currently in clinical use (Fig. 3.1) (Rates, 2001; Thomford et al., 2018). The 19th century witnessed a boom in pharmacologically active molecules isolated from higher plants with morphine from *Papaver somniferum* L. (Papaveraceae), being the first marketed plant-based drug. This launched a new era of pharmaceutical interest in plants. The antiprotozoal and emetic drug, emetine, was isolated from the *Cephaelis ipecacuanha* (Brot.) A. Rich (Rubiaceae) in 1817 (Grollman & Jarkovsky, 1975) followed by the antimalarial quinine from the bark of *Cinchona ledgeriana* (Howard) Bern. Moens ex Trimen (Rubiaceae) in 1820 and the cholinergic drug pilocarpine isolated from *Pilocarpus jaborandi* Holmes (Rubiaceae) in 1875 (Horne, Fugmann, Yakushijin, & Büchi, 1993) amongst others. Plants have provided numerous pure active compounds or synthetically optimized analogues of high therapeutic value. Fifteen of the 56 natural product-derived oncology drugs approved since 1980 originated from plants with a long medicinal history (Willis, 2017). Adding to this, analysis of the WHO essential medicines list revealed that 11% of the listed drugs are exclusively derived from plants (Rates, 2001). Many of these molecules remain vital in the fight against life-threatening diseases. For instance the cardiac glycoside, digoxin, isolated from *Digitalis lanata* Ehrh (Plantaginaceae), used in the management of atrial fibrillation in cardiac disease, had an estimated global sales value of US$142 million for the year 2012 and was identified among the top 10 most prescribed drugs in the United States (IARC, 2016). Moreover, artemisinin, and its derivatives originating from the plant *Artemisia annua* (Asteraceace), play a vital role in curbing the global malaria crisis and were estimated to account for about 22% of the 663 million prevented clinical cases (Su & Miller, 2015). The discovery of two natural product-derived antimalarial agents, namely artemisinin and avermectin, a bacterial-derived agent, was acknowledged by the 2015 Nobel prize for medicine, highlighting the tremendous impact of these pharmaceuticals in public health (Su & Miller, 2015). Strikingly, modern science can only

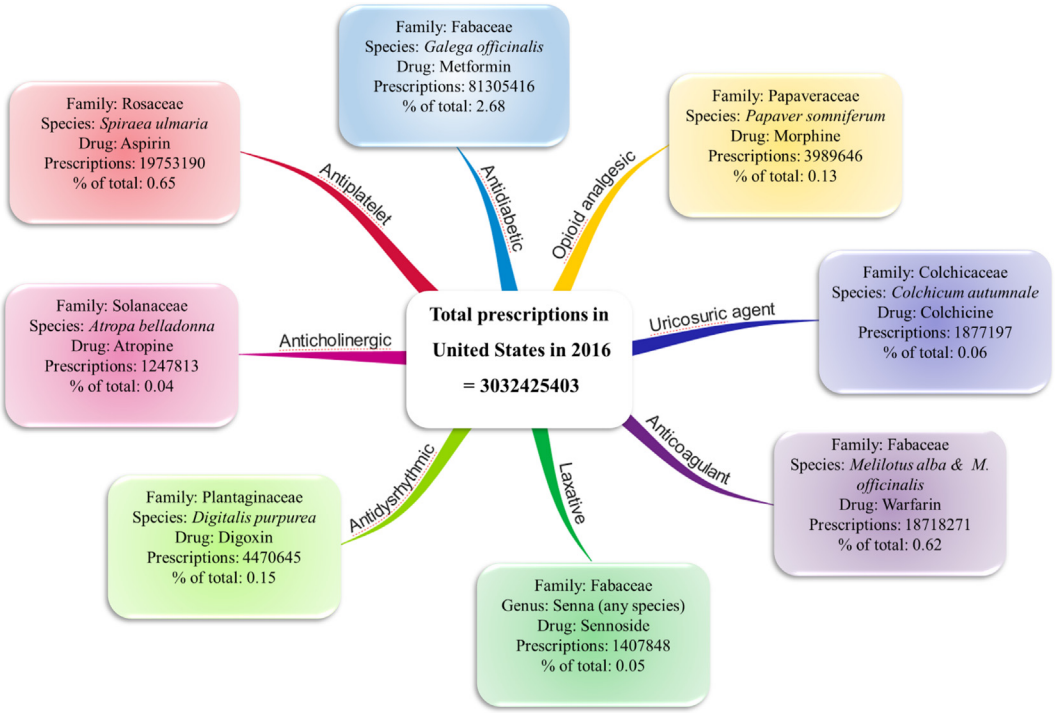

FIGURE 3.1 Plant-derived drugs and their total prescriptions in the United States for the year 2016. *Data from ClinCalc DrugStats Database. (2019).* ClinCalc drug stats database. *Retrieved June 15, 2019, from <https://clincalc.com/DrugStats/>.*

take partial credit, as properties of *A. annua* were identified in Chinese traditional medicine. Adding to this, metformin, the discovery which stemmed from the medicinal plant *Galega officinalis* L. (Fabaceae), is used as the first line of treatment against type 2 diabetes and is estimated to influence 125 million people worldwide (Rojas & Gomes, 2013; Triggle & Ding, 2017). These two examples highlight the need to preserve and study traditional medicine as a source for new drugs.

As evidenced by the plant-derived blockbuster clinical agents, including artemisinin, paclitaxel, morphine, and metformin, natural products continue to prevail as a fertile reservoir to probe for novel therapeutic agents (Su & Miller, 2015; Thomford et al., 2018; Triggle & Ding, 2017). Over 120 agents undergoing some form of preclinical or clinical trials have natural products, that is, originating from microbes, marine organisms, or plants, as their starting point (Newman, 2018). Furthermore, 45% of the clinical drugs approved worldwide, including in Canada, India, the United Kingdom, the United States, Japan, the European Union, and Taiwan, in the year 2010 alone originated from natural products (Newman, 2018). Likewise, 20% and 26% of the approved clinical agents in these countries for the year 2016 and 2017, respectively, were derived from natural products (Newman, 2018). The first botanical formulation approved by the US Food and Drug Administration (FDA), in 2006, was an antiviral topical formulation of sinecatechins, comprising a blend of eight different green tea catechins and other green tea components, for the treatment of external genital and perianal warts (Kleindl et al., 2017; Tyring, 2012). Six years later, the FDA approved another botanical drug named crofelemer for the treatment of HIV-associated diarrhea in HIV/AIDS patients (Kleindl et al., 2017). Crofelemer consists of a proanthocyanidin structure and is extracted from the *Croton lechleri* (Euphorbiaceae) plant (Kleindl et al., 2017). A recent addition to the existing plant-derived armamentarium includes the oral solution of crystalline cannabidiol, purified from *Cannabis sativa* (Cannabaceae) and approved by the FDA in 2018 for the treatment of seizures (Traynor, 2018; Yang & Szaflarski, 2019).

3.4 New prospects in drug discovery from plants

A large body of published data suggests that despite the drastic loss of biodiversity and hence chemical diversity on the planet, the remaining heritage would afford solutions to most if not all known and emerging conditions (Díaz, Fargione, Chapin, & Tilman, 2006; Rummun, Neergheen-Bhujun, Pynee, Baider, & Bahorun, 2018). Although the production levels of drugs from plants as well as the threatened status of the plant species are two major limitations in the drug

discovery process, alternative solutions, such as metabolic engineering and microbial production platforms, provide much optimism (Brower, 2008). In addition, the increasing applications of updated technologies like high-throughput screening, transcriptomic, proteomic, and metabolomic analyses, synthetic chemistry, and precision analytical instrumentation add more value to the search for bioactive compounds from plants because these approaches can identify metabolic pathways from plants with potentially useful compounds (Chakraborty, 2018; Chen, Xiang, Guo, & Li, 2011; Kellner et al., 2015). The potential of repurposing existing drugs also plays a potential role in curbing the effects of a declining number of new drugs coming on the market (Janes et al., 2018). Major research efforts have been directed toward plant-derived small molecules that were showing promise of being repurposed for use as anticancer drugs. For instance, metformin is currently being developed in several clinical trials both synergistically with other drugs like taxanes and also individually against various cancer types (Sleire et al., 2017). In addition, more than 10 drugs have been repurposed for neuropsychiatric disorders (Lee & Kim, 2016).

Maximizing the benefit of plant biodiversity to human health is anticipated due to the rapid advancement of informatics and data management. For example, mapping chemistry, traditional and modern medicine on plant phylogenetic history would help predict the most promising plants and chemistries and greatly assist drug development. Augmented reality technologies in conjunction with satellite photosurveillance and ecological niche molding should allow us to build global dynamic interactive maps of existing plant resources, and predict major changes resulting from changes in climate, industrial, and agriculture development. Finally, further advancement of distributed ledger technologies (e.g., blockchain) promises to overcome the legislative crisis related to access, benefit sharing, and intellectual property. Fair and transparent distribution of benefits from medicinal plants, coordinated with native communities in developing countries, whilst at the same time being attractive for pharmaceutical companies and academic research laboratories, is imperative for identifying new molecules from plants.

3.5 Current context of barriers

The research and development of natural products is currently regulated by the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (Convention on Biological Diversity, 2019). This international agreement aims at sharing the benefits arising from genetic resources in a fair and equitable way. It entered into force on October 12, 2014 but it has been controversial because of its complication in many aspects which would not be easy for many developing countries with small research laboratories and limited expert resources.

Rather than creating a more fair and equitable research on natural products, the Nagoya protocol has made it extremely difficult for small research laboratories to research and develop natural products in the countries that follow the agreement (Rourke, 2018). It has created barriers for research, especially for smaller research laboratories that often cannot afford to or cannot efficiently navigate the requirements to initiate research (Lawson, Humphries, & Rourke, 2019; Watanabe, 2015). For instance, for the protocol's success, it would require effective implementation at the domestic level with a belief that a range of tools and mechanisms will assist the contracting parties. These include, but are not limited to, establishing national focal points (NFPs) and competent national authorities (CNAs) to serve as contact points for information, grant access or corporate on issues of compliance, and an access and benefit sharing (ABS) clearing house to share information such as domestic regulatory ABS requirements or information on NFPs and CNAs. Although this is considered effective in many countries with well-equipped infrastructure, it may not be the case in the developing world where management of databases and the subject matters like genetic resources is relatively weak. In some countries in Southeast Asia, such as Indonesia, Malaysia, and Thailand, the information related to genetic resources, plants, and patents are handled under different government agencies and ministries with limited cooperation and communication between each other.

Under the ABS concept, prior informed consent (PIC) and mutually agreed terms (MAT) are required for any use of genetic resources. With respect to PIC, it covers a process where foreign researchers are required to get a permit to access genetic resources of a member state. For biodiversity hot spot countries like Indonesia, the procedure to get a research permit for foreign researchers is complex (Latifah, 2015). There is no one-stop service provided and that leads to multiple layers of institutional consideration prior to the granting of any permit. Further, when it involves "benefit sharing," oftentimes the provider, such as a national authority, would expect to receive the sharing benefit in a monetary ratio. Whether commercial and noncommercial research has the grounds to identify benefit sharing could be another challenge. These days, many public research organizations and universities are encouraged to file patent protection on their inventions. A patent application is known as one of the key performance indicators (KPIs) that affects their career development. When determining whether basic research is for commercial use, a patent application is deemed to be a

key indicator of potential commercialization. Nonetheless, the KPIs do not reflect much commercial viability for researchers in the developed world.

Thus without clear guidelines on benefit sharing and what constitutes commercialization, this requires significant amounts of time that eventually delays the progress of research. As for MAT, whether the agreed terms are deemed fair, and what form of legal document constitutes MAT under the Nagoya protocol are in doubt. For intellectual property rights based on genetic resources to be registered domestically under the patent system, a certificate of disclosure of origin issued by the competent national authority must be submitted (Medaglia, Perron-Welch, & Phillips, 2014). Without a transitional period, it would be difficult for researchers to have the required support documents in place.

3.6 Conclusion: approaches to circumventing the barriers and challenges

Tackling the aforementioned challenges will require continuous integration and cooperative engagement of policy makers, researchers, social scientists, and members of indigenous communities worldwide. Several action teams have been recently established, including the Commission on Planetary Health by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), Rockefeller Foundation (Planetary Health commission), Digital Forest (started in Brazil to evaluate genomic and metabolomic diversity in the Amazon), and the Biodiversity for Survival via Biomedicine (Bio2Bio) consortium started within the Global Young Academy (Neergheen-Bhujun et al., 2017). Engagement with these initiatives can serve as a platform to highlight the urgency of biodiversity protection for biomedical applications and to lay out a strategic plan going forward.

Hence, the authors who are from the Bio2Bio consortium of the Global Young Academy propose that there is an opportunity to create new policies aimed at the sustainable protection of bio- and molecular diversity on the basis of our own survival. The databases available at GBIF and IUCN provide a foundation for this information hub and the library should be combined with a multilingual database portal, which would allow patients, researchers, and medical professionals to find up-to-date scientific information on relevant compounds. The database should also include details on the medicinal value of compounds that occur within common and endangered species, local or endemic foods, traditional medicine, and a phylogeny to create predictions for new species for potential future research and compound development. This should nevertheless necessitate appropriate measures to protect these resources. Secondly, since humanity's pursuit of a valuable commodity has driven valuable species to extinction and destroyed habitats that support diverse species, it is imperative that communities are educated on the responsible use of biodiversity for medicinal purposes. In addition, making the aforementioned technologies and training available to all countries in the quest for drug discovery from plants whilst maintaining intellectual property rights will require that stakeholders work with policy makers in the global policy space to create new policies that partner researchers, industries, and local communities with socially just and equitable sharing models.

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Chapter 4

Biomining fungal endophytes from tropical plants and seaweeds for drug discovery

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4.1 Introduction

The resilience of disease-causing agents to readily available chemotherapeutic drugs has raised global concern. Many diseases are now becoming resistant to many of our available drugs. Cancer is now the leading cause of death worldwide and is considered as one of the global challenges of the 21st century (World Health Organization, 2018a). According to the World Cancer Report, the global incidence of cancer rose to an estimated 14 million new cases in 2012, and this figure is expected to rise to an annual 19.3 million cases by 2025 (Stewart, and Wild, 2014). In the Philippines, the breast cancer incidence is among the highest in Asia (Laudico et al., 2015; World Health Organization, 2014). Despite the availability of chemotherapeutic drugs, the treatment for cancer is made even more challenging by the occurrence of multidrug resistant (MDR) cancer cells (Mansoori, Mohammadi, Davudian, Shirjang, & Baradaran, 2017). Likewise, increasing antimicrobial resistance (AMR) is also of equal global concern. Bacterial species, like *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*, are becoming resistant to antibiotics due to the acquisition of different virulent factors (World Health Organization, 2018b). With the global concern for the prevalence of diseases and increased resistance to chemotherapeutic agents, there is an unyielding need to find novel chemicals from untapped biological resources and perform high-throughput screening for target-specific biological activities to identify promising compounds that can be subjected to clinical testing (Demain, 2014).

Nowadays, the scientific community is interested in investigating a variety of microorganisms, mainly bacteria and fungi, to address world health problems. An intensive search for newer antimicrobial agents is recognizing the use of endophytic fungi as potential sources of bioactive secondary metabolites (Bungihan et al., 2010; Ghadin et al., 2008; Maria, Sridhar, & Raviraja, 2005; Radu & Kqueen, 2002). In fact, over 25% of prescription drugs used in human medicine are obtained from endophytic fungi that reside within plant tissues (Aly, Debbab, & Proksch, 2011; Li et al., 2011; Shai, McGaw, Masoko, & Eloff, 2008). Moreover, studies on various marine organisms and their associated microbiomes led to the isolation of around 25,000 metabolites (Blunt, Copp, Keyzers, Munro, & Prinsep, 2015). Indeed, the bioprospecting of natural products from marine and terrestrial endophytes is an emerging scientific pursuit (Aly et al., 2011; Strobel & Daisy, 2003).

Many of the compounds isolated from fungal endophytes exhibited novel pharmacological activities against selected viruses, Gram-positive and Gram-negative bacteria, fungi, and cancer cell lines (Newman & Cragg, 2014). In the past, the production of bioactive secondary metabolites was solely attributed to the metabolic machinery of the host organisms. However, recent discoveries showed that the metabolic pathways responsible for the synthesis of these previously identified bioactive chemicals result from the coadaptation and mutualistic interaction between the host organisms and

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their microbial associates. On top of that, it is also possible for horizontal gene transfer to occur between the hosts and their endosymbionts, further enhancing the chemodiversity of the metabolites they produce (Strobel, 2018). Given their metabolic diversity, marine and terrestrial fungal endophytes are indeed of great value to science and medicine.

4.2 Endophytic fungi from terrestrial plants

Tropical plants harbor a wide array of endophytic fungi. In the Philippines, the endemic plant *Canarium ovatum* is host to endophytic fungi belonging to the following genera: *Acremonium*, *Alternaria*, *Aureobasidium*, *Coprinus*, *Chaetomium*, *Colletotrichum*, *Culvularia*, *Fusarium*, *Geotrichum*, *Guignardia*, *Myrothecium*, *Nigrospora*, *Paecilomyces*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phialophora*, and *Rhizoctonia* (Torres & dela Cruz, 2015). Solis, dela Cruz, Schnittler, and Unterseher (2016) also studied the leaf-inhabiting fungal endophytes associated with fig trees and listed 22 genera from three species of *Ficus—F. religiosa*, *F. benjamina*, and *F. elastica*. Their study noted that the host plant influenced the community structure of the fungal endophytes. In a separate study, dos Banhos et al. (2014) reported the diversity and bioactivity of endophytic fungi from a tropical plant, *Myrcia guianensis*, collected in Brazil. They were able to isolate *Pestalotiopsis*, *Xylaria*, *Fusarium*, *Penicillium*, *Nectria*, *Phomopsis*, and *Aspergillus*. Wanasinghe et al. (2018) also identified 93 new endophytes belonging to 17 new genera from *Rosa* (Rosaceae) in Thailand. The new genera identified were *Bhatiellae*, *Italica*, *Cycasicola*, *Dactylidina*, *Hawksworthiana*, *Uzbekistanica*, *Embarria*, *Melanocucurbitaria*, *Monoseptella*, *Melanodiplodia*, *Neoconiothyrium*, *Neopaucispora*, *Pararoussoella*, *Marjia*, *Paraxylaria*, *Sporormurispora*, and *Xenomassariosphaeria*. *Colletotrichum* and *Phyllosticta* were also isolated from *Citrus* plants (Glienke-Blanco, Aguilar-Vildoso, Vieira, Barroso, & Azevedo, 2002). The diversity of plant-associated fungi makes these microorganisms ideal candidates for drug discovery.

In the study of Isaka, Berkaew, Intereya, Komwijit, and Sathitkunanon (2007), their research focused on the identification of bioactive compounds from local fungi in Thailand and investigated the constituents of the endophytic fungus *Pullularia* sp. isolated from the leaf of *Culophyllum* sp. Their research showed that the cyclohexadepsipeptides, pullularins A−D, had modest cytotoxicity against cancer cell lines like KB cells (oral human epidermoid carcinoma), BC cells (human breast cancer), and NCI-H187 (human small cell lung cancer). The compounds also showed antimalarial activity against *Plasmodium falciparum* K1, antiviral activity against the human herpes simplex virus type 1 (HSV-1), and antimycobacterial activity against *M. tuberculosis* H_{37}Ra. Moreover, the endophytic fungi from *M. guianensis* were also assayed against pathogenic microorganisms. Results showed that *Nectria haematococca* was the best source of antimicrobial compounds with MIC values of 50 μg/mL and 100 μg/mL against *S. aureus* and *Penicillium avellaneum*, respectively (dos Banhos et al., 2014). *Phomopsis cassiae*, a fungal endophyte associated with the legume *Cassia spectabilis*, contained cadinane sesquiterpenoids, namely 3,9,12-trihydroxycalamenene, 3,12-dihyroxycalamenene, 3,12-dihydroxycadalene, and 3,11,12-trihydroxycadalene. Among these, 3,11,12-trihydroxycadalene was shown to have the highest antifungal activity against *Cladosporium* sp. (Silva et al., 2006). Antifungal activity can also be observed against plant pathogens of their host plant. For example, in the study of Dagamac, Sogono, Cabalfin, Adducul, and dela Cruz (2008), root fungal endophytes isolated from *Musa* spp. were antagonistic to *Fusarium oxysporum*, a causative agent of vascular wilt disease in banana. In addition to antimicrobial properties, antioxidant activities were also exhibited by fungal endophytes associated with three Philippine medicinal plants, that is, *Gliricidia sepium*, *Canna indica*, and *Gardenia jasminoides* (Eskandarighadikolaii, dela Cruz, & Bungihan, 2015). These biological activities have demonstrated the potential of fungal endophytes for bioprospecting of pharmacologically active chemical constituents.

The continuous exploration of fungal endophytes resulted in other host plants being studied for promising metabolites. Hussain, Krohn, Ullah, Draeger, and Barbara (2007) investigated several endophytic fungi from various plant species for the isolation of chemical constituents with different biological activities like antifungal, antibacterial, and antioxidant properties. The group isolated djalonensone from the endophytic fungus *Acremonium* sp. associated with *Plantago lanceolata*. This compound showed strong antifungal activity against the phytopathogenic fungus *Microbotryum violaceum*. Lu, Zou, Meng, Hu, and Tan (2000) also isolated an endophyte from the stem of *Artemisia annua*. This was identified as *Colletotrichum* sp. and was found to produce the secondary metabolites, 3β,5α-dihydroxy-6β-acetoxy-ergosta-7,22-diene and 3β,5α-dihydroxy-6β-phenylacetyloxy-ergosta-7,22-diene. These metabolites showed antifungal activity against *Candida albicans* and *Aspergillus niger*, as well as against other different crop-associated pathogenic fungi. On the other hand, Liu et al. (2004) identified eight bioactive secondary metabolites from *Aspergillus fumigatus*, an endophytic fungus in *Cynodon dactylon*. Among the eight isolates, asperfumoid, fumigaclavine C, fumitremorgin C, physicon, and helvoic acid inhibited *C. albicans* in vitro. Strobel et al. (2002) also reported the isolation of isopestacin, an isobenzofuranone obtained from the endophytic fungus *Pestalotiopsis microspora*. While a few other isobenzofuranones are known from other natural sources, isopestacin is the only one having a substituted

benzene ring attached at the C-3 position of the furanone ring. The compound was isolated from culture broths of the fungus and was crystallized, with its structure determined by X-ray crystallography. This compound possesses antifungal activity and, as measured by electron spin resonance spectroscopy, behaves as an antioxidant scavenging both superoxide and hydroxyl free radicals.

With the occurrence of multidrug-resistant strains of *M. tuberculosis*, researchers also found the urgent need for the development of new drugs from plants and microorganisms that target this infectious bacterium. Several secondary metabolites were isolated from endophytic fungi that targeted tubercle bacilli. Among these are the new pimarane diterpenes, diaportheins A and B, isolated from the culture broth of the fungus *Diaporthe* sp. BCC 6140. These compounds showed inhibitory activity against *M. tuberculosis* (Dettrakul et al., 2003). Rukachaisirikul, Sommart, Phongpaichit, Sakayaroj, and Kirtikara (2008) identified five secondary metabolites from *Phomopsis* sp. These compounds were phomoenamide, phomonitroester, deacetylphomoxanthone, dicerandrol, (1*S* − 2*S* − 4*S*)-*p*-menthane-1,2,4-triol, and uridine. Phomoenamide also showed bioactivity against *M. tuberculosis* H_{37}Ra in vitro.

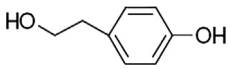
4.3 Endophytic fungi from the *Pandanaceae*

Owing to the diversity of plants in the tropics, it is interesting to look at how a particular plant family is host to fungal endophytes and could be sources of novel chemicals. The *Pandanaceae* family is found mainly in the tropical countries and comprises five genera, namely, *Pandanus*, *Freycinetia*, *Sararanga*, *Martellidendron*, and *Benstonea*. The *Pandanus* are usually trees or shrubs, the *Freycinetia* are climbers, and the *Sararanga* are trees. Meanwhile, *Martellidendrons* are more closely related to the *Freycinetia* than *Pandanus* (Callmander, 2001; Callmander, Chassot, Kupfer, & Lowry, 2003). *Benstonea* is the latest addition to the *Pandanaceae* genera. The genus *Pandanus* is the biggest among the four genera. Several bioactive endophytic fungi were isolated from *Pandanus* and *Freycinetia*. To date, there are no published studies on the endophytic fungi from the other three genera.

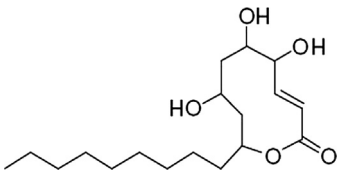
In a review of the fungus *Anthostomella* from palms and *Pandanus*, five taxa were found to be occurring on *Pandanus*. These are *A. baileyi*, *A. lucens*, *A. minutoides*, *A. pandani*, and *A. phoenicicola*. In addition to these species, *Pandanus rigidifolius* was found to harbor *A. petrinensis* while *A. theobromina* was isolated from *Pandanus palustris* and *Pandanus eydouxia* (Dulymamode, Cannon, & Peerally, 1998a). Endophytes coming from the genus *Astrocystis* were also found in the same *Pandanus* species. *A. fimbriata*, *A. rarissima*, and *A. cepiformis* are usually associated with the dead leaves of *Pandanus*. Particularly, *A. fimbriata* was described from its host *P. eydouxia* and *A. rarissima* in *P. palustris*, while *A. cepiformis* was found in both plants (Dulymamode, Cannon, & Peerally, 1998b). The genus *Linocarpoon* was also found to be endemic endophytes within the mentioned *Pandanus* species, including *P. rigidifolius* and *P. barklyi*. Specifically, *L. elaeidis* is found in *P. rigidifolius*, *L. fasciatum* in *P. eydouxia*, *L. spathulatum* in *P. palustris*, and *L. sulcatum* in both *P. barklyi* and *P. rigidifolius* (Dulymamode, Cannon, & Peerally, 1998c). Moreover, ascomycetes from *Pandanus* spp. were also identified. *Lepteutypa tropicalis* was isolated from the tissues of *P. rigidifolius* and *P. palustris*, while *Pellucida pendulina* was identified in *P. palustris*, *P. eydouxia*, and *P. barklyi* (Dulymamode, Cannon, Sivanesan, & Peerally, 2001a). In the study of Dulymamode, Cannon, and Peerally (2001b), the mycobiota isolated from the monocotyledonous genus *Pandanus* is more distinct than those from the dicotyledonous hosts *Sideroxylon*, *Cordemoya*, and *Olea*. Many isolates were from well-known genera such as *Anthostomella*, *Astrosphaeriella*, *Astrocystis*, *Linocarpon*, *Niesslia*, and *Sporidesmium*. Three endophytic fungi, *Englerodothis oleae*, *Pseudorhynchia mauritiana*, and *Rubikia splendida*, were new additions to the monotypic genera. In the Philippines, Bungihan, Nonato, Dareger, Franzblau, and dela Cruz (2013) also isolated and identified 28 morphospecies of endophytic fungi from *Pandanus amaryllifolius*. Some of the identified species belong to the genera *Colletotrichum*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Phoma*, *Phyllosticta*, *Trichoderma*, *Chaetomium*, *Diaporthe*, *Lulworthia*, and *Truncatella*. Moreover, Tibpromma et al. (2018) established taxonomic keys for the identification of endophytic fungi from the Pandanaceae family from two genera, *Pandanus* and *Freycinetia*, in Thailand. The endophytic fungi were identified using both morphological and molecular data. In *Pandanus* spp. the following endophytes were isolated and identified: *Alternaria burnsii*, *Chryseobacterium endophyticum*, *Chionaspis pandanicola*, *Colletotrichum fruticola*, *Diaporthe pandanicola*, *Daboia siamensis*, *Epermenia thailandica*, *Lasiodiplodia theobromae*, *Menisporopsis pandanicola*, *Meyerozyma caribbica*, *Plaudibacter jiangxiensis*, *P. microspore*, *Phanerochaete chrysosporium*, and *Phyllosticta capitalensis*. Tibpromma et al. (2018) also reported the isolation of *Endomelanconiopsis freycinetiae*, *Martelella endophytica*, *Epermenia thailandica*, and *C. fruticola* from *Freycinetia*. Comparison of the number of species isolated from species of *Pandanus* and *Freycinetia* showed that there was a greater number of fungal endophytes living within *Pandanus*. All these studies illustrate that a single family of plants can be a host to a variety of fungal endophytes.

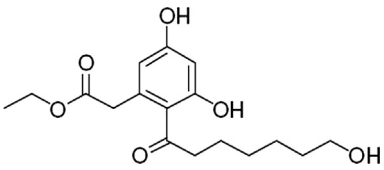
Aside from the diversity of the endophytes from the *Pandanus* plants, several bioactive chemicals were reported from these microorganisms. For instance, the study of Bungihan et al. (2011) with fungal endophytes associated with *P. amaryllifolius* led to the discovery of novel bioactive compounds identified through bioassay-guided isolation. Two novel compounds from *Diaporthe* sp. P133, namely, diaportheone A and diaportheone B, showed inhibitory activity against a virulent strain of *M. tuberculosis* H37Rv with minimum inhibitory concentrations of 100.9 and 3.5 μM, respectively.

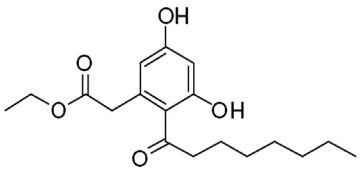
In addition, two antioxidant compounds, guignardiol from *Guignardia* sp. (Bungihan et al. (2010) and tyrosol [1] from *Colletotrichum gloeosporioides* (Bungihan, Tan, Takayama, dela Cruz, & Nonato, 2013) were isolated and structurally identified. Ahn et al. (2008) established the antioxidant and anticancer activities of tyrosol and its derivatives which were found to be promising inhibitors of functional DNA replication proteins such as topoisomerase I and pol α-primase.



Another macrolide antibiotic, colletotriolide [2] from *C. gloeosporioides* and dothiorelone C [3] and cytosporone B [4] from *C. globosum* showed antibacterial activities against *E. coli* (Bungihan et al., 2013). Cytosporone B, in particular, rendered an IC_{50} of 62.5 μg/mL against the said bacterial pathogen.







4.4 Endophytic fungi from estuarine and marine plants

The vast biological resources in the ocean make it a conducive reservoir for many kinds of organisms. There are about 2.2−3.8 million species of fungi worldwide (Hawksworth & Lücking, 2017). Among these immensely diverse microorganisms are the marine-derived fungi (MDF) that form an ecological rather than a taxonomic group (Raghukumar, 2008). Kohlmeyer and Kohlmeyer (1979) classified MDF as either obligate or facultative. Obligate marine fungi are those that exclusively grow and sporulate in the marine waters, while the facultative ones are those derived from freshwater or terrestrial *milieu* that have acquired the ability to grow and sporulate in the marine environment. MDF which are endophytic in nature thrive within healthy tissues of marine plants without eliciting any disease or harm. Endophytes derived their nutrients from the host plants and, in exchange for their cohabitation, the endophytes enhance the host plants' overall growth and development, resilience to different environmental stressors (including pathogenic attacks), and the ability to better assimilate nutrients from the environment. This mutualistic coexistence between the host plant and the endophytic microorganism is essential for the plant's survival and evolution (Kusari, Hertweck, & Spiteller, 2012). Current monographic

estimates showed that there were a total of 530 species in 321 genera of filamentous MDF. This includes 424 species of ascomycetes in 251 genera, 94 species of anamorphic fungi in 61 genera, and 12 species of basidiomycetes in 9 genera (Jones, Sakayaroj, Suetrong, Somrithipol, & Pang, 2009). However, this figure only applies to MDF isolated and characterized based on culture-dependent methods. With the advent of molecular research, there is a pronounced disparity in terms of the biological diversity evident from culture-dependent methods as compared to the diversity based from molecular studies that rely on the gene sequences found in the environmental samples. Hence, the actual diversity of MDF is expected to be much higher than what is reported (Rateb & Ebel, 2011).

There are a number of factors that influence the growth of fungi in the marine ecosystem and these include salinity, pH, temperature, and pressure. The tolerance of MDF to salinity, which on average is at 33−35 ppt in seawater, is one determining factor of their growth and colonization to various substrates (Raghukumar, 2008). Further, high concentration of sodium ions in seawater may be toxic to the cells of terrestrial and freshwater organisms. MDF overcome this unwanted toxicity by sequestering sodium ions in vacuoles or by having an efficient sodium efflux ATPase (Benito, Garciadeblás, & Navarro, 2002). As compared to terrestrial fungi that usually grow at pH 4.5−6, facultative MDF produce extracellular enzymes that enable them to grow at pH 7−8 (Raghukumar et al., 1994). Moreover, mycelial fragments in comparison with fungal spores are more tolerant to elevated hydrostatic pressure and low temperature in the deep sea. Exposure of microorganisms to various environmental stressors may lead to the biosynthesis of stress proteins like heat shock, cold shock, or antifreeze proteins that enable them to survive in hostile environment (Raghukumar, Damare, & Singh, 2010). These physiological adaptations are crucial for the enhancement of the chemodiversity of MDF that enable them to produce unprecedented and biotechnologically relevant chemicals that cannot be synthesized from terrestrial sources. Since environmental conditions dictate the type of metabolites that organisms produce in their natural ecosystem, this makes MDF promising sources of drugs and other natural products. As evidently shown in the study of Schulz et al. (2008), fungal endophytes from marine habitats were equally good sources of novel metabolites, albeit the ratio of metabolites to fungal taxon and the proportion of novel metabolites were higher for fungi isolated from plants than those isolated from marine algae. It is also interesting to note in this study of Schulz et al. (2008) that metabolites produced by fungi isolated from marine plants and algae had diverse structures, 42% of which were previously unknown.

Aside from marine plants, mangroves which inhabit the estuarine ecosystem also harbored fungal endophytes with many diverse biologically active secondary metabolites. Huang et al. (2008) reported the isolation of three metabolites, namely phomopsins A, B, and C, together with two known compounds, cytosporone B and C, from the mangrove endophytic fungus *Phomopsis* sp. ZSU-H76 obtained from the South China Sea. Cytosporone B and C inhibited two fungi, *C. albicans* and *F. oxysporum*, with an MIC ranging from 32 to 64 μg/mL. Tan, dela Cruz, Apurillo, and Proksch (2015) identified tyrosol C, cytosporone B, dothiorelone A, and dothiorelone C from culture extracts of *Phyllosticta* sp. isolated from a mangrove in the Philippines. These metabolites were previously reported to have antibacterial, anticancer, and antioxidant properties (Ahn et al., 2008; Bungihan et al., 2013).

Recognizing the importance of mangroves as hosts to fungal endophytes capable of producing novel and structurally diverse metabolites, the authors compared the diversity and biological activities of fungal endophytes isolated from seven mangrove hosts—*Rhizophora stylosa*, *Sonneratia alba*, *Sonneratia caseolaris*, *Avicennia marina*, *Lumnitzera racemosa*, *Excoecaria agallocha* and *Acanthus ebracteatus*—collected in two megadiverse countries, Indonesia and the Philippines (Fig. 4.1). From a total of 351 fungal endophytes, we observed a high number of fungal isolates and morphospecies in *S. caseolaris* and *A. marina*, respectively (Table 4.1). Clearly, the host and the sampling locality had an effect on the number of fungal endophytes. Nevertheless, this exemplified the enormous opportunities for the biodiscovery of fungal endophytes in tropical mangrove ecosystem.

Equally promising are the metabolites produced by these mangrove-associated fungi. A simple and easy-to-do thin-layer chromatographic (TLC) profiling showed differences in rf values, indicating different metabolic profiles (Fig. 4.2), although in the study of Schulz et al. (2008), geographic location had little effect on the metabolic profiles of

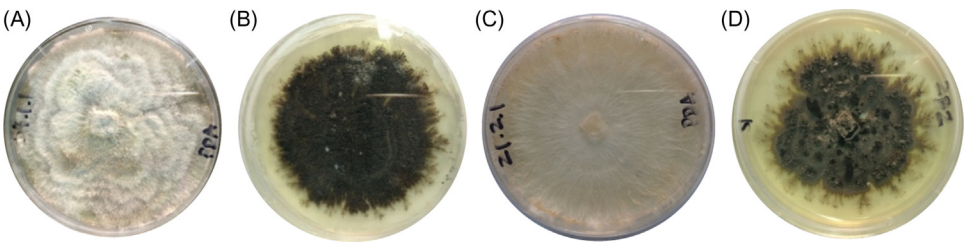


FIGURE 4.1 Examples of mangrove fungal endophytes isolated from Indonesia (A, B) and the Philippines (C, D).

TABLE 4.1 Diversity of fungal endophytes associated with different mangrove hosts from Indonesia and the Philippines.

Locality	Sampling sites	Host mangroves	Number of morphospecies	Number of fungal isolates
Indonesia	Barru	*Rhizophora stylosa*	1	6
		Sonneratia alba	2	28
	Segeri	*Rhizophora stylosa*	2	4
		Sonneratia alba	2	23
		Excoecaria agallocha	4	44
		Lumnitzera racemosa	3	53
	Marros	*Rhizophora stylosa*	2	8
Philippines		*Avicennia marina*	1	1
	Bolong	*Sonneratia caseolaris*	3	23
		Avicennia marina	3	32
		Acanthus ebracteatus	3	21
	Cawit	*Sonneratia caseolaris*	5	70
		Avicennia marina	7	36

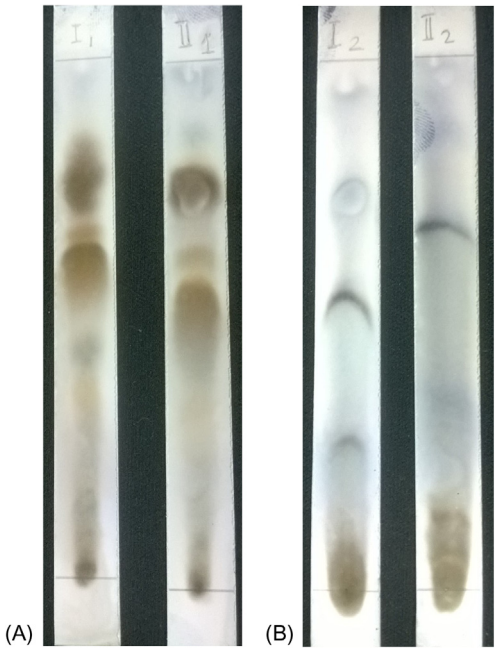


FIGURE 4.2 TLC chromatogram of the crude culture extracts produced by mangrove fungal endophytes: (A) from Indonesia; (B) from the Philippines.

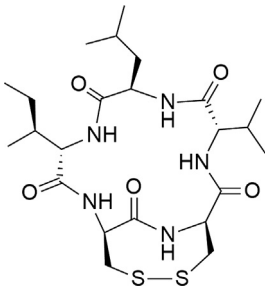
saprobic, marine-derived fungi. In the same study, geographic origin did influence the proportion of crude culture extracts with antifungal activities. Dela Cruz, Wagner, and Schulz (2006) also noted that the production of bioactive metabolites by marine-derived fungi was strain-specific. The biological activities exhibited by mangrove-associated fungi are also evident in the study of Moron, Lim, and dela Cruz (2018). In their paper, the 22 fungal endophytes isolated from the 12 mangrove hosts showed antimicrobial activities against at least one of the test bacteria and fungi with *P. microspora* showing strong inhibitory activity against Gram-positive bacteria and species of *Lasiodiplodia* as very active against yeasts. Apurillo, Cai, and dela Cruz (2019) also showed the antibacterial and cytotoxic activities of 16

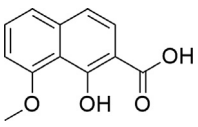
fungal endophytes isolated from mangroves. A closely related taxon, *Pestalotiopsis adusta* showed very promising activity against *P. aeruginosa* with an MIC of 80 μg/mL. Their paper also noted the effect of incubation condition (agitated vs. static) on the cytotoxic activities of mangrove fungal endophytes. Interestingly, even decomposing mangrove leaves can be hosts to fungi with the ability to produce extracellular enzymes of biotechnological significance (Torres & dela Cruz, 2013).

4.5 Endophytic fungi from seaweeds

The discovery of penicillin fueled the exploration and exploitation of filamentous fungi as excellent sources of antibiotics; development of cephalosporin into a marketed drug and many other recent studies have demonstrated the potential of antimicrobials with vast chemodiversity from marine fungi (Silber, Kramer, Labes, & Tasdemir, 2016). Despite the absence of marine fungal-derived metabolites in the current clinical anticancer drug pipeline, many of them have been classified as potential chemotherapy candidates because of their anticancer activity (Gomes, Lefranc, Kijjoa, & Kiss, 2015). Interestingly, the Philippines is an epicenter of marine diversity with an estimated 820 species of macroalgae (Trono, 1999) and 16 species of seagrasses (Fortes, 2013). Meanwhile, Indonesia which is 1083 miles away from the Philippines is also a home to 11 species of seagrasses and 117 species of macroalgae (Verheij & Erftemeijer, 1993). This enormous diversity of macroalgae and seagrasses represents an exhaustive host for endophytic fungi, many of which can be potentially novel species that can be tapped for the discovery of promising chemotherapeutic agents.

In the study of Notarte, Yaguchi, Suganuma, and dela Cruz (2018), a total of 16 morphospecies of marine-derived fungi were isolated from four host macroalgae and two seagrasses collected from the Negros Island in the Central Visayas, Philippines. These were classified as belonging to the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sclerotinia*, and *Thamnidium*. Some of these MDF, for example, *Aspergillus* and *Fusarium*, showed antibacterial and cytotoxic properties. On top of that, *Aspergillus tubingensis* isolated from the seagrass *Enhalus acoroides* afforded the known cyclic pentapeptide malformin A_1 [5] that was potent against HeLa cervical (IC_{50}: 50.15 ng/mL) and P388 murine leukemia (IC_{50}: 70.38 ng/mL) cancer cell lines, and the parasite *Trypanosoma congolense* with IC_{50} at 15.08 ng/mL (Notarte, Nakao, Yaguchi, Suganuma, & dela Cruz, 2017). Moreover, Lavadia, Dagamac, and dela Cruz (2017) isolated 29 morphospecies of algicolous fungi from macroalgae collected from Potipot Island and Lubang Island, Northern Philippines. The algicolous fungi were identified as *Aspergillus*, *Alternaria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Nigrospora*, *Pestalotia*, *Penicillium*, and *Trichoderma*. Among the algal groups, brown algae showed the highest fungal species diversity. Interestingly, the crude extracts of the algicolous fungi successfully reduced biofilm formation of *S. aureus* as much as ≥99%. On the other hand, in the study of Solis, Draeger, and dela Cruz (2010), algicolous fungi can contribute to secondary infection, particularly the *ice-ice* disease, among cultivated seaweeds. This *ice-ice* disease has an enormous economic consequence, particularly on seaweed farmers.





In the study of Tarman et al. (2011), they studied the fungal endophytes associated with Indonesian macroalgae. Three fungal strains from *Kappaphycus alvarezii*, two strains from *Eucheuma edule*, and one strain from *Gracilaria* were isolated. These strains belonged to the genera *Aspergillus*, *Lasiodiplodia*, *Epicoccum*, *Xylaria*, and *Coniothyrium*. Among the isolates, the ethyl acetate extract from the sterile fungal strain KT31 derived from *K. alvarezii* showed

cytotoxicity against human bladder carcinoma cell line 5637 (IC_{50}: 1.5 μg/mL). In the same study, the sterile fungal strain KT29 afforded the novel compound 2-carboxy-8-methoxy-naphthalene-1-ol [6] which is an intermediate in the synthesis of a naphthalene carboxylic acid using naphthalenediol as a starting material. In contrast to other naphthalene derivatives, this new natural product did not show significant antimicrobial and cytotoxic properties. In another study, Tarman et al. (2012) also isolated the endophytic fungus *Daldinia eschscholzii* associated with *Gracilaria* and afforded the fungistatic novel compound helicascolide C and the structurally related known compound helicascolide A. Despite the pharmacological significance of seagrass and algicolous fungi, very few studies have been carried out in the Asia Pacific region, particularly the Philippines and Indonesia. Thus it will be a fruitful endeavor to study these seagrass-associated and algicolous fungi from the region.

4.6 Emerging techniques for optimizing the metabolic potential of fungal endophytes

One major challenge in the use of microbial resources, including fungi for drug discovery, lies in artificial fermentation conditions, which are not identical to their natural habitats and often with missing environmental signals. Indeed, recent genomic sequencing studies showed that the biosynthetic potential of many fungal strains is much greater than that observed by fermentation. This indicates that gene clusters possessed by fungi encode for the production of a large variety of compounds, but they remained undetected as their microbial biosynthetic genes remain silenced under standard, single-strain laboratory culture conditions (Netzker et al., 2015). Hence, classical single-strain fungal natural products' chemistry work reflects only a little portion of the diversity of fungal metabolites. This also leads to the report of a limited number and types of metabolites, many of which are already known to the literature. The induction of the silent biosynthetic pathways at the earliest (culture) stage of the drug discovery process to produce previously unknown, unusual metabolites with potentially higher biological activity profiles represents a new strategy to overcome the increasing difficulty in discovering new bioactive structures.

Another promising approach to address this problem is the so-called OSMAC (One-Strain-MAny-Compounds), a simple but effective method for monocultural microbial fermentations (Bode, Bethe, Höfs, & Zeeck, 2002). OSMAC seeks to identify the most suitable culture media and other conditions for optimal fungal growth, which also determines the type (and the amount) of secondary metabolite biosynthesis. The effects of varying the culture conditions were also observed in algicolous and seagrass fungi. In fact, Notarte et al. (2018) reported that cultivating marine aspergilli in solid rice media led to a weaker or loss of bioactivity against selected cancer cell lines as opposed to those cultivated in liquid media like potato dextrose broth and malt extract broth with or without marine salt supplementation. It is also worth noting that *A. tubingensis* IFM 63452 isolated from the seagrass *E. acoroides* showed better trypanocidal and anticancer properties when cultivated in the presence of marine salt regardless of the culture medium used during cultivation. In a separate study by Tarman et al. (2011) algicolous fungal strain KT31 showed better anticancer property when grown in freshwater culture affording an IC_{50} of 1.5 μg/mL as opposed to the seawater culture that afforded an IC_{50} of 14 μg/mL. The manipulation of the cultivation parameters, for example, the composition/type of culture media, pH, temperature, the addition of epigenetic modifiers such as histone deacetylase (HDAC) inhibitors, or UV mutagenesis, are applied to naturally induce the expression of certain genes/gene clusters and signal transduction pathways that have been restricted during the traditional monoculturing of a fungal species (Paranagama, Wijeratne, & Gunatilaka, 2007). Interestingly, addition of dietary components from fruits and herbs in culturing fungal endophytes has also been proven to activate their cryptic metabolic pathways. For instance, Sharma et al. (2017) reported that supplementing grape skin and turmeric extracts in the liquid cultures of *C. gloeosporioides* could lead to the production of additional secondary metabolites that were previously not synthesized by the fungus. Even more intriguing is that the crude extract derived from the treated fungus showed much more enhanced antibacterial and antioxidant properties as compared to the controls not exposed to the grape skin and turmeric extracts.

Another strategy for increasing the chemical diversity of fungal species is mixed fermentation (also known as coculture), that is, the growth of a microbial species in the presence of another microbe(s). The rationale behind the coculturing approach relies on the creation of a competitive microbial ecosystem, ideally similar to that found in their natural environment, to induce a competitive response between microorganisms (Pettit, 2011). The resulting chemical interactions mediated via interspecies cross-talks and the expression of small molecules are expected to stimulate cryptic biosynthetic pathways, thereby causing induced production of novel types of secondary metabolites or previously unknown analogues of a known compound(s). Although it is still an emerging strategy, coculturing has already yielded chemically new and interesting compounds with antibiotic and also anticancer activities (Bertrand et al., 2014; Müller & Wink, 2014). In one study

by Ola, Thomy, Lai, Brötz-Oesterhelt, and Proksch (2013), *Fusarium tricinctum* was cocultured with the Gram-positive *Bacillus subtilis* 168 trpC2 on solid rice medium. This effort resulted in the 78-fold increase in the synthesis of constitutively present secondary metabolites. One of these metabolites is lateropyrone that demonstrated wide-spectrum antibacterial activities against *B. subtilis*, *S. aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*. Furthermore, the detection of new natural products is also aided with the advances in analytical techniques. Using imaging mass spectrometry (IMS), it is now possible to locate the spatial organization of naturally produced secondary metabolites which can be used to further understand the role of these chemicals in the interaction between microorganisms. In fact, the application of IMS has been proven vital for the discovery of the tolasin metabolites and the novel virulence factor jagaricin which are both inducers of brown blotch mushroom disease (Netzker et al., 2015). Another advancement in the analytical method involves the detection of coculture-derived natural products using liquid chromatography-mass spectrometry (LC-MS). For instance, Bertrand et al. (2013) used LC-MS to analyze the metabolome of more than 600 coculture experiments, involving mainly fungi of the *Fusarium* genera, leading to the discovery of new molecular masses that were previously nonexistent in natural product databases. In addition, LC-MS also paved the way for the isolation of new antibiotics such as alchivemycin A (Onaka, Mori, Igarashi, & Furumai, 2011).

As vital as the advancement of analytical tools for detecting new compounds is the use of the "omics" technology that also plays an important role in studying the metabolic potential of microorganisms. Organismal characterization at the DNA, RNA, and protein levels as well as the identification of the regulons for the expression of these molecules provide a more comprehensive insight into the genomic and the metabolic state of the organism of interest (Monaghan & Barrett, 2006). The application of these molecular techniques is best exemplified by the study of Mao et al. (2015) wherein genome sequencing of a mushroom-derived endophytic fungus, *Calcarisporium arbuscula*, revealed 68 core genes that are vital in natural product biosynthesis. Interestingly, epigenetic modification induced by the inactivation of a histone H3 deacetylase led to the overexpression of more than 75% of the biosynthetic genes from this fungus and eventually paved the way for the isolation of novel cyclic peptides such as arbumycin and arbumelin. Furthermore, there has also been an increasing effort to metabolically engineer some filamentous fungi using a clustered regulatory interspaced short palindromic repeat/Cas9 system to increase the production of lovastatin and taxol, considering that the clusters of genes that encode these metabolites remained cryptic under standard laboratory cultural conditions (El-Sayed, Abdel-Ghany, & Ali, 2017). Metagenomics and genome editing also aid in determining the hidden chemodiversity of unculturable endophytic fungi. Transfecting DNA from environmental samples to a competent host strain, for example, *Saccharomyces cerevisiae*, could cater to the production of novel compounds. Through heterologous expression of silent gene or gene clusters and the molecular modification of biosynthetic pathways, optimization of a natural product is therefore achievable (Silber et al., 2016).

4.7 Concluding remarks

In the paper of Neergheen-Bhujun et al. (2017), the authors call for the protection of biodiversity. They argued that protecting and preserving biodiversity is very crucial, if not the most important step, in achieving the 17 Sustainable Development Goals (SDG) set by the United Nations. The authors further argued that the development of future drugs is intertwined with biodiversity preservation. Any activities that threaten biodiversity, for example, habitat loss, habitat degradation, global pollution, climate change, etc., will surely have a tremendous impact on our efforts to discover new metabolites against multidrug-resistant infectious agents and other life-diminishing diseases. And quoting the very words of Neergheen-Bhujun et al. (2017), "the preservation of biodiversity provides a vital link to critically expand the molecular diversity necessary for successful drug discovery efforts in the future." The archipelagic countries, Indonesia and the Philippines, are two of the 18 megadiverse countries worldwide. These countries are home to a vast number of species of flora and fauna, many of which are associated with other organisms such as fungi, that can be harnessed for their enormous chemical diversity. The goal we must set therefore is to continuously explore, document, study, and preserve our countries' biodiversity for the discovery of bioactive compounds.

4.8 Acknowledgments

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Chapter 5

Biomedicine developments based on marine biodiversity: present and future

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5.1 Marine biodiversity

The Mediterranean coasts have witnessed the flourishing and decline of many civilizations, as the crossroads of Africa, Europe, and Asia. The Mediterranean Sea connects through the Strait of Gibraltar to the Atlantic Ocean in the west and through the Dardanelles to the Sea of Marmara and the Black Sea in the northeast. In the southeast, the Suez Canal links the Mediterranean to the Red Sea and the Indian Ocean.

Generally, evaporation is higher in the eastern half of Mediterranean basin, while the annual mean sea surface temperature shows a high seasonality and important gradients from west to east and north to south. The Mediterranean basin is generally oligotrophic, but regional features enrich coastal areas through changing wind conditions, temporal thermoclines, currents and river discharges, and municipal sewage (Bosc, Bricaud, & Antoine, 2004; Estrada, 1996; Zavatarelli, Raicich, Bregant, Russo, & Artegiani, 1998), and the biological production decreases from north to south and west to east and is inversely related to the increase in temperature and salinity.

The Mediterranean has narrow continental shelves and a large area of open sea and includes some unusual features: (1) high homothermy from 300 to 500 m to the bottom and (2) high salinity of 37.5−39.5 psu. There are no thermal boundaries in the deep sea of the Mediterranean, unlike the Atlantic Ocean, where temperature decreases with depth. Shelf waters represent 20% of the total Mediterranean waters and therefore play a proportionally greater role here than in the world's oceans (Pinardi, Arneri, Crise, Ravaioli, & Zavatarelli, 2006).

The recent marine biota in the Mediterranean Sea is primarily derived from the Atlantic Ocean, but the wide ranges of climate and hydrology have contributed to the cooccurrence and survival of both temperate and subtropical organisms (Bianchi & Morri, 2000; Sara, 1985). High percentages of Mediterranean marine species are endemic (Tortonese, 1985). This sea also has its own set of emblematic species, such as sea turtles, several cetaceans, and the critically endangered Mediterranean monk seal (*Monachus monachus*), while it remains the main spawning grounds of the eastern Atlantic bluefin tuna (*Thunnus thynnus*) (Bearzi, Holcer, & Notarbartolo di Sciara, 2004; MacKenzie, Mosegaard, & Rosenberg, 2009; Reijnders, Verriopoulos, & Smj, 1997. There are several unique and endangered habitats, including the seagrass meadows of the endemic *Posidonia oceanica*, coralligenous assemblages (Ballesteros, 2006; Goren & Galil, 2001; Green & Short, 2003), and deep-sea and pelagic habitats that support unique species and ecosystems (Sardà, Calafat, Flexas, Tselepides, & Canals, 2004; Sardà, Company, Rotllant, & Coll, 2009).

Recently, Coll and colleagues' (2010) analysis revealed that approximately 17,000 species occur in the Mediterranean Sea, with at least 26% prokaryotic (Bacteria and Archaea) and eukaryotic (Protists) marine microbes, though the data available for Bacteria, Archaea, and Protists were very limited, together with the data corresponding to several invertebrate groups (such as Chelicerata, Myriapoda, and Insecta). Within the Animalia, the greater proportion of species records were from subphylum Crustacea (13.2%) and phyla Mollusca (12.4%), Annelida (6.6%), Platyhelminthes (5.9%), Cnidaria (4.5%), the subphylum Vertebrata (4.1%), Porifera (4.0%), Bryozoa (2.3%), the subphylum Tunicata (1.3%), and Echinodermata (0.9%). Other invertebrate groups encompassed 14% of the species, and Plantae included 5%. Available information (Coll et al., 2010) showed that the highest percentage of endemic species

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was in Porifera (48%), followed by Mysidacea (36%), Ascidiacea (35%), Cumacea (32%), Echinodermata (24%), Bryozoa (23%), seaweeds and seagrasses (22%), Aves (20%), Polychaeta (19%), Pisces (12%), Cephalopoda (10%), and Decapoda (10%).

In the detailed analyses performed by Coll et al. (2010), an important bulk of species diversity was attributed to the prokaryotic (Bacteria and Archaea) and eukaryotic (Protists) marine microbes, though they accepted the fact that the continuously changing state of the knowledge of marine microbial diversity made it difficult to provide species estimates for the Mediterranean (or from anywhere else) and establish comparisons.

Predators are the species that occupy the upper trophic levels, normally beyond the level of secondary consumers, which have lower diversity than other taxonomic groups. Coll et al. (2010) reviewed data available for fish, seabirds, marine mammals, and turtles in the Mediterranean Sea. Generally, Mediterranean scientific institutes are actively performing several censuses of marine mammals or turtles, normally by using transect data collected from aerial or boat-based sighting surveys developed to assess abundance, while movement patterns are tracked with transmitters and monitored by satellite tracking as well. Fish species are mainly studied using scuba diving or fishing techniques. According to Coll et al. (2010), several estimates of Mediterranean diversity exist regarding fish species: Hofrichter (2002) summarizes 648 species, and Golani, Orsi Relini, Massuti, and Quignard (2002) report a total of 650 fishes occurring in the Mediterranean Sea. The updated list of exotic fish species (CIESM, 2009) reveals that the Mediterranean currently contains 116 exotic species. Approximately 80 fish species are elasmobranchs, although the status of some is uncertain because of infrequency or uncertain reporting (Cavanagh & Gibson, 2007; Compagno, 2001; Serena, 2005). According to Cavanagh and Gibson (2007), nine of these elasmobranch species may not breed in the Mediterranean, while some are rare because the Mediterranean represents the edge of their distribution ranges. Only four batoid species are Mediterranean endemics: the Maltese skate (*Leucoraja melitensis*), the speckled skate (*Raja polystigma*), the rough ray (*Raja radula*), and the giant devilray (*Mobula mobular*) (Serena, 2005). Nine species of marine mammals are encountered regularly in the Mediterranean (Frantzis, Alexiadou, Paximadis, Politi, & Gannier, 2003; Reeves & Notarbartolo di Sciara, 2006), five belong to the Delphinidae, and the others belong to the Ziphiidae, Physeteridae, Balaenopteridae, and Phocidae, respectively.

According to Pérès (1985), the deepwater fauna of the Mediterranean has a lower degree of endemism than that of the Atlantic at similar depths. So while the Mediterranean basin is recognized as one of the most diverse regions on the planet, the deep sea in the Mediterranean may contain a much lower diversity than deep-sea regions of the Atlantic and Pacific oceans (Lambshead, Brown, Ferrero, Mitchell, & Smith, 2002; Lambshead, Tietjen, Ferrero, & Jensen, 2000). Macpherson (2002) and Briggs (2007) have suggested that within the Atlantic−Mediterranean region, the fauna (including invertebrates and fishes) of the Mediterranean is more diverse than that of the Atlantic and displays considerable endemism. In the deep sea of the Mediterranean, small-bodied taxa can reach a high diversity, and with the presence of a high prokaryotic diversity in the sediments of the deep-sea Mediterranean (Danovaro, Corinaldesi, Luna, Magagnini, & Manini, 2009), this may change the view that the Mediterranean deep-sea biota is impoverished in comparison with its Atlantic counterpart. Endemic macrobenthic species account for approximately 13%−15% of total species number at depths from 200 to 1000 m, and approximately 20% at 2000 m (Bellan-Santini, Fredj, & Bellan, 1992). New species in different sectors of the deep Mediterranean (Danovaro, Company, Corinaldesi, D'Onghia, & Galil, 2010) show that the general conclusion that the biodiversity is high in coastal systems and low in the deep sea of the Mediterranean might not hold true (Danovaro et al., 2010).

The biodiversity of the Mediterranean is definitively influenced by the introduction of new species (Galil, 2006, 2007; Galil, Gollasch, Minchin, & Olenin, 2009; Streftaris & Zenetos, 2006; Streftaris, Zenetos, & Papathanassiou, 2005; Zenetos, Çinar, Pancucci-Papadopoulou, Harmelin, & Furnari, 2005; Zenetos, Meriç, Verlaque, Galli, & Boudouresque, 2008; Zenetos, Pancucci-Papadopoulou, Zogaris, Papastergiadou, & Vardakas, 2009). More than 600 metazoan species have been recorded as alien and all these species most likely to be introduced by the predominant pathways (the Suez Canal, vessels, and mariculture) are shallow-water species.

Diversity distribution in the Mediterranean is associated with a productivity gradient. Higher productivity areas show higher diversity, partially because they are important feeding and reproductive sites for several taxa. Most of these areas occur in the Western Mediterranean and the northern Adriatic, which host many species of fish, seabirds, marine mammals, and turtles (Cañadas, Sagarminaga, & Garc a-Tiscar, 2002; Mínguez, Oro, de Juana, & Martinez-Abrain, 2003). Their distribution is associated with feeding habits (Bearzi, Agazzi, Gonzalvo, Costa, & Bonizzoni, 2008; Bearzi, Fortuna, & Reeves, 2008; IUCN/UNEP, 1988). Moreover, some fish, seabirds, sea turtles, and mammals show opportunistic feeding behavior, exploiting discards from trawling and purse seines, and to a lesser extent from artisanal long-lining (Bozzano & Sardà 2002; Oro & Ruiz, 1997; Tomas, Aznar, & Raga, 2001). Most Mediterranean marine mammals are predominantly offshore and prefer deepwater habitats, but a few species can venture to inshore waters

and scavenge fishery discards (Cañadas et al., 2002; Cuttelod, García, Abdul Malak, Temple, & Katariya, 2008; Reeves & Notarbartolo di Sciara, 2006).

5.2 Threats to biodiversity

The general patterns and temporal trends of Mediterranean marine diversity with varying degrees of intensity have been influenced by anthropogenic factors (Jackson, Kirby, Berger, Bjorndal, & Botsford, 2001). Published information and the opinion of experts identified and ranked current threats to diversity in the Mediterranean (Fig. 5.1). The sum of the ranking (0−5 for each threat) showed that for 13 large taxonomic groups, habitat loss and degradation are considered the primary impact on diversity, followed by exploitation, pollution, climate change, eutrophication, and species invasions (Coll et al., 2010). Within 10 years from now, habitat degradation and exploitation were predicted to retain the predominant roles, while pollution and climate change will likely increase in importance, followed by eutrophication. Of all current threats to biodiversity in the Mediterranean, climate change was predicted to show the largest growth in importance within the next 10 years (10.8%), followed by habitat degradation (9.2%), exploitation (6.2%), and pollution, eutrophication, and invasion of species (4.6% each)

Taking into account data regarding marine biodiversity and threats, Coll and colleagues (2010) mapped vertebrate endangered species and tried to locate potential hot-spot areas of special concern for conservation in the Mediterranean. The first attempt included fish, marine mammals, and sea turtles, which are considered important sentinels for ocean health. The identified hot spots highlighted the ecological importance of most of the western Mediterranean shelves. The Strait of Gibraltar and the adjacent Alboran Sea and African coast were identified as representing important habitat for many threatened or endangered vertebrate species. Both the northern Adriatic and Aegean seas also showed concentrations of endangered, threatened, or vulnerable species. Other equally species-rich waters along the northeast African coast, and the southern Adriatic Sea, were of lesser concern for the protection of endangered species.

Coll et al.'s (2010) results showed that habitat degradation and loss is currently the most widespread threat, where human interventions, such as coastal modification, have important consequences for diversity. Coastal development, sediment loading, and pollution reduced the extent of important habitats for marine diversity, such as seagrass meadows, and affected Mediterranean ecosystem functioning. Most species depend strongly on their habitats (such as bryozoans, sponges, echinoderms, benthic decapods, and organisms of the suprabenthos and meiobenthos); hence, its loss and degradation have major effects on marine diversity. Direct and indirect pollution is generated directly from the coast, or through fluvial contributions, and ends up in the sea (Bas, 2009). Pollution affects a wide range of marine species (Borrell, Aguilar, & Pastor, 1997; Giangrande, Licciano, & Musco, 2005; Hummon, Todaro, Balsamo, & Tongiorgi, 1990; Ruffino, Bourgeois, Vidal, Duhem, & Paracuellos, 2009; Sanpera, Moreno, Ruiz, & Jover, 2007) and

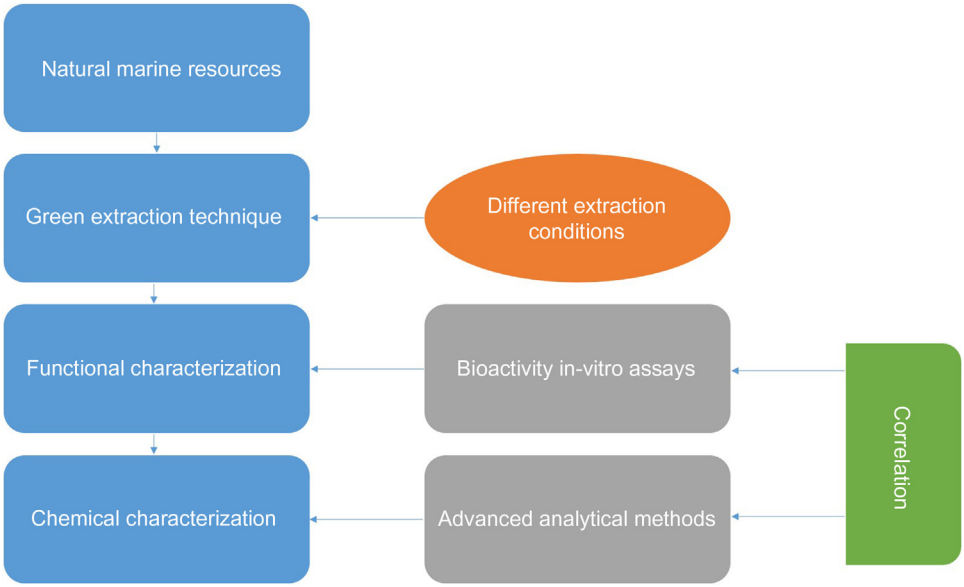

FIGURE 5.1 Basic scheme showing the proposed workflow for the screening of bioactive compounds from marine sources.

is of primary concern for the conservation of the deep-sea ecosystems (Danovaro et al., 2010). Marine wind farms, which are expected to increase in some countries, may represent a new conservation concern for seabird populations (Garthe & Hüppop, 2004). Marine turtles are also affected primarily by degradation of habitats but also by marine pollution, driftnets, gillnet and longline bycatches, and boat strikes (Camiñas, 2004; Groombridge, 1990; Margaritoulis, 2000). The continuing increase of coastal settlements is important for the region's economic activity, but it is also causing intense environmental degradation through excessive coastal development, further pollution, and consumption of natural resources, all of which add pressure to coastal areas and the marine environment (Hofrichter, 2001).

The current high demand for marine products has resulted in high levels of fishing or harvesting intensity. Several fish resources are highly exploited or overexploited (Bas, Maynou, Sardà, & Lleonart, 2003; Bombace & Grati, 2007; FAO, 2004; Lleonart & Maynou, 2003; Papaconstantinou & Farrugio, 2000; MacKenzie et al., 2009). Other organisms that are exploited or affected by exploitation in the Mediterranean include macrophytes, sponges, cnidarians, echinoderms, mollusks, arthropods, polychaetes, ascidians, and other invertebrates (Pronzato & Manconi, 2008; Sardà 1998). The threats to currently endangered marine mammals and sea turtles include unwanted bycatch (IUCN, 2009; Tudela, 2004) as well as historical exploitation. For sea turtles, the overall mortality rate caused by entanglement in fishing gear and by habitat degradation is poorly known (Camiñas, 2004), but for marine mammals the major threats clearly derive from human activities: direct or indirect effects of exploitation, such as prey depletion, direct killing, and fishery bycatch (Aguilar & Raga, 1993; Bearzi, Agazzi, et al., 2008; Bearzi, Fortuna et al., 2008; Reeves & Notarbartolo di Sciara, 2006; Reeves, Smith, Crespo, & Notarbartolo di Sciara, 2003; Tudela, Kai, Maynou, El Andalossi, & Guglielmi, 2005). At sea, threats to seabirds mainly come from fisheries (Arcos, Louzao, & Oro, 2008; Louzao, Igual, McMinn, Aguilar, & Triay, 2006), particularly bycatch in longlining (Igual, Tavecchia, Jenouvrier, Forero, & Oro, 2009). Fishing is being expanded toward deeper areas and is threatening several ecosystems (Coll, Palomera, & Tudela, 2009; Libralato, Coll, Tudela, Palomera, & Pranovi, 2008), while management effectiveness in the Mediterranean is low (Mora, Myers, Coll, Libralato, & Pitcher, 2009; Tsikliras, Moutopoulos, & Stergiou, 2007). Fishing activity may also be the cause of ecosystem structural and functional changes and ecosystem degradation (Coll, Lotze, & Romanuk, 2008; Guidetti, Terlizzi, Fraschetti, & Boero, 2003).

5.3 Marine biomedicine

The science in which marine organisms are used to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses is named Marine Biotechnology. Humans have been able to elucidate many biological methods applicable to both aquatic and terrestrial organisms, with the help of different molecular and biotechnological techniques.

The marine environment may contain over 80% of world's plant and animal species (McCarthy, & Pomponi, 2004) and many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs, and other marine organisms (Donia & Hamann, 2003; Haefner, 2003). Furthermore, the deep knowledge about nerve transmission has been learnt using squid and its giant nerve axons, while the mesenteries of vision have been unraveled using the eyes of horseshoe crabs, sharks, and skates.

The marine environment is one of the most underutilized biological resources. In the literature, algae and microalgae are referenced as sources of bioactive compounds for use as functional food ingredients (Plaza, Cifuentes, & Ibañez, 2008; Plaza, Herrero, Cifuentes, & Ibañez, 2009). The huge diversity of macro- and microalgae, coupled with the hostile environments in which these organisms live, make macro- and microalgae key targets for bioactive compound screening projects.

Algae comprise a complex and heterogeneous group of organisms characterized by their photosynthetic nature and their simple reproductive structures. Generally, algae live in extreme environments of light, salinity, and temperature, where most algae produce a high variety of secondary metabolites (often have potent biological activities), in order to adapt to these extreme conditions.

Bioactive compounds have also been isolated previously from other marine organisms including crustaceans, fish, and their by-products. In some of these matrices, interesting functional compounds were isolated previously (Kadam & Prabhasankar, 2010; Kim & Wijesekara, 2010).

Considering the great biodiversity of marine species, the use of appropriate methodologies that can rapidly screen different marine sources for bioactive compounds is of great interest. Different parameters have to be considered in order to design this screening methodology. These parameters include the possible nature of the sought-after bioactive compounds (in terms of solubility, heat resistance, or molecular weight) and the bioactivity that is sought. Fig. 5.1 proposes a screening methodology for the extraction and identification of bioactive compounds from marine sources.

Initially, a suitable extraction technique should be selected. This selection must be carried out in accordance with the predicted nature of the expected/target bioactive compound(s). However, several extraction techniques could also be used to fully characterize the potential of the different natural sources, introducing different extraction selectivity.

The use of environmentally clean advanced extraction techniques allows for the attainment of the target compound(s) of interest with more efficient extraction procedures, while, at the same time, minimizing the use of organic toxic solvents.

Diverse extraction parameters should be tested in order to study the influence of solvents, temperatures, pressures, and other important parameters that might have a significant influence on the outcome of the extraction process employed, depending on the selected extraction techniques. The different extracts, obtained using diverse conditions, must then be tested for biological bioactivities by performing the appropriate functional activity assay(s). The main aim of this step is to confirm that the obtained extracts from step one possess the sought-after bioactivity.

Functional characterization should be assessed through the application of fast in vitro assays directed to the confirmation of the sought biological properties, for instance, antioxidant capacity assays, antimicrobial activity assays, or antihypertensive activity assays. Once the target biological activities have been confirmed, the next step involves chemical characterization of the bioactive components present in the initial extract, which may often be referred to as lead functional components (LFCs). The analytical technique will depend on the nature of the initial extract or LFC in terms of its solubility, stability at different pH conditions, and heat stability, as well as the nature of the suspected bioactive compounds, employed at this stage of the characterization process.

In general, advanced analytical techniques are employed, even coupled, in order to maximize the identification potential. The final aim of this stage of the characterization process is the correlation between the chemical composition of the LFC and the bioactivities observed. Further use of the information gathered using this integrated approach can be directed toward the design of upscale procedures for this extraction and characterization.

Despite the fact that the biodiversity in the marine environment far exceeds that of the terrestrial environment, research into the use of marine natural products as pharmaceutical agents is still in its infancy. This may be due to the lack of ethnomedical history and the difficulties involved in the collection of marine organisms (Faulkner, 1992). Actually, with the development of new diving techniques and remote operated machines, it is possible to collect marine samples and during the past decade, over 5000 novel compounds have been isolated from shallow waters to 900-m depths of the sea (McCarthy, & Pomponi, 2004).

Marine organisms have developed into very sophisticated physiological and biochemical systems during evolution. During the adaptation to the terrestrial environment, a number of physiological changes have taken place, but in most cases the biological systems have almost completely retained the basic functions. The architecture of the shark liver is similar to that of the human liver and the shark's biochemical transformations appear to be similar to those that occur in a human liver (Wolf, 1978), with slight modifications (Halvey, 1990). The eyes of man and octopus are very similar in structure and function, irrespective of the fact that no evolutionary link exists between them (Salisbury, 1971). Insulin from fish such as cod exerts the same hormonal activity in mammals as does homologous insulin and insulin from tuna (which has a 40% difference in amino acid residue (Grant & Mackie, 1977)) that has been used to treat diabetic patients (Halvey, 1990). This suggests that the basic physiological functions of molecules may remain the same regardless of the structural changes, which may possibly occur during evolution (Halvey, 1990).

The knowledge of the physiological and biochemical features of marine organisms might contribute to the identification of natural products of biomedical importance.

In spite of many successes in drug discovery from microorganisms, marine microorganisms have received very little attention. The difficulty in the search of metabolites from marine bacteria is mainly due to the nonculturability of the majority (over 99%) (Hugenholtz & Pace, 1996).

Marine toxins such as tetrodotoxin, saxitoxin, ciguatoxins, and brevetoxins are potent and specific sodium channel blockers, and pharmacological studies with these toxins have played a major role in developing the concept of sodium channels in general and membrane channels and voltage-gated sodium channels in particular (Auyoung, 1999; Dechraoui, Naar, Pauillac, & Legrand, 1999; Kao & Levinson, 1986). Several studies show that these toxins may be produced by marine bacteria (Kodama, Ogata, & Sato, 1988; Kodama, Ogata, Sato, & Sakamoto, 1990; Simudu, Kita-Tsukamoto, Yasumoto, & Yotsu, 1990). These toxins are useful in neurophysiological and neuropharmacological studies, and marine bacteria could be an important source of these valuable molecules.

Important and promising metabolites have been isolated and further analyzed in sponges, cnidarians, mollusks, echinoderms, and fish species. Approximately 10,000 sponges have been described in the world and most of them live in marine waters. A range of bioactive metabolites has been found in about 11 sponge genera. Three of these genera (Haliclona, Petrosia, and Discodemia) produce powerful anticancer, antiinflammatory agents, but their cultivation has

not been studied (Blunt, Copp, Munro, Northcote, & Prinsep, 2004). An aminoacridine alkaloid, dercitin, has been isolated from the deepwater sponge, *Dercitus* spp., which possesses cytotoxic activities in the low nanomolar concentration range, and in animal studies prolongs the life of mice-bearing ascitic P388 tumors, and resulted in also being active against B16 melanoma cells and small cell Lewis lung carcinoma (Burres, Sazech, Gunavardana, & Clement, 1989). Two new -pyrones (herbarin) along with a new phthalide, herbaric acid, were isolated from two cultured strains of the fungus *Cladosporium herbarum* isolated from the sponges *Aplysina aerophoba* and *Callyspongia aerizusa* collected in the French Mediterranean and in Indonesian waters, respectively (Judulco et al., 2002). Herbarins displayed activity in the brine shrimp assay (Judulco et al., 2002).

The discovery of prostaglandin in corals in the late 1960s contributed greatly to the rapid developments in the field of marine natural products (Carte, 1996). Palytoxin is the product of *Palythoa* species of the family Zoanthidae and it represents one of the most potent known toxins. It is a useful tool for probing cellular recognition processes since it stimulates arachidonic acid metabolism and downregulates the response to epidermal growth factor by activating a sodium pump in the signal transduction pathway using sodium as the second messenger. Bioassay-guided fractionation of extracts obtained from soft coral, *Lobophytum crassum*, indicated ceramide to be a moderately antibacterial component (Vanisree & Subbaraju, 2002).

More than 2600 scientific studies over the last 20 years testify to the important contribution to medicine and cellular biology of toxins extracted from cone snails. Roughly 100 out of a potential 50,000 toxins have been extracted and analyzed. The *Conus* species have evolved deadly nerve toxins and small, conformationally constrained peptides of 10−30 amino acids. Some of the conotoxins block channels regulating the flow of potassium or sodium across the membranes of nerve or muscle cells; others bind to N-methyl-D-aspartate receptors to allow calcium ions into nerve cells; and some are specific antagonists of acetylcholine receptors responsible for muscle contraction. Thus conotoxin are valuable probes in physiological and pharmacological studies (Myers, Cruz, Rivier, & Olivera, 1993). A team from the University of Melbourne extracted the conotoxin from a cone-shell snail. It not only inhibits pain, being 10,000 times more powerful than morphine, but also accelerates the recovery of injured nerves (Holmes, 2002). The absolute stereochemistries of membrenones A−C, -dihydropyrone-containing polypropionates isolated from the skin of the Mediterranean mollusk *Pleurobranchus membranaceus*, have been determined by stereocontrolled syntheses of the enantiomers (Sampson & Perkins, 2002).

Bursatellanin-P, a 60-kDa protein was purified from the purple ink of the sea hare *Bursatella leachii* (Rajaganapathi, Kathiresan, & Singh, 2002). The protein exhibited anti-HIV activity. The first total syntheses of aplyolides B−E, ichthyotoxic macrolides isolated from the skin of sea hare, *Aplysia depilans* (Spinella, Zubía, Martinez, Ortea, & Cimino, 1997), have been reported confirming the absolute stereochemistry reported for the metabolites (Cañadas et al., 2002; Spinella, Caruso, & Coluccini, 2002).

Physiologically active saponins have been studied extensively from sea stars and sea cucumbers (Dubois, Higuchi, Komori, & Sasaki, 1988), but they are not so useful as drugs because of their tendency to cause cell lysis. Even then, glycosylated ceramides and saponins continue to be the major classes of metabolites identified in echinoderms. A full account of the isolation and characterization of hedathiosulphonic acids A and B, isolated from a deep-sea urchin *Echinocardium cordatum* (Takada et al., 2001), has been reported by Kita et al. (2002).

Metabolites extracted from fish, sea snakes, and aquatic mammals are scanty. Various fish species are used to extract fish oil, rich in omega-3 fatty acids, which is used in the preparation of various kinds of drugs for the remedies of human beings, such as arthritis and many others.

Throughout the world about 500 species of fish are considered toxic. The most spectacular substance of pharmacological importance extracted from fish is tetradotoxin (TTX), the puffer or fugu poison. Other toxins isolated include ciguatoxin from electric rays, which is served as a potent antidote for pesticide poisoning (Oliviera et al., 2003). TTX isolated from puffer fish and many other marine organisms has become a useful tool for researchers studying the voltage-gated sodium channel, and tetradotoxin also plays an important role in many biological experiments.

There has been great interest in analyzing fish antimicrobial peptides, with the main purpose of using them in aquaculture and biomedicine. During the first stages of their lives, when the adaptive immunity is still not active, and when they are completely developed, fishes rely heavily on their innate immune defenses for initial protection against invasion of pathogen agents, as the adaptive immune system displays scarce memory and short-lived secondary responses (Du Pasquier, 2001). The antimicrobial peptides (AMPs) are one of the major components of the innate defences in protecting from such infections. In mammals, AMPs typically have broad-spectrum antimicrobial activity, they can often kill multiple pathogens that include bacteria, fungi, parasites, and viruses. A large number of AMPs have been isolated from a wide number of fish species during recent years, among which are pleurocidin from winter flounder (*Pleuronectes americanus*) (Cole, Weis, & Diamond, 1997), cathelicidins from rainbow trout (*Oncorhynchus mykiss*)

(Chang, Pleguezuelos, Zhang, Zou, & Secombes, 2005), defensins from zebrafish (*Danio rerio*) (Zou, Mercier, Koussounadis, & Secombes, 2007), piscidins from hybrid striped bass (white bass, *Morone chrysops*, female, x striped bass, *Morone saxatilis*, male) (Silphaduang & Noga, 2001), dicentracin from sea bass (*Dicentrarchus labrax*) (Salerno, Parrinello, Roch, & Cammarata, 2007), hepcidin from channel catfish (*Ictalurus punctatus*) (Bao, Peatman, Li, He, & Liu, 2005), and epinecidin from the grouper (*Epinephelus coiodes*) (Pan, Chen, Cheng, Chen, & Ni, 2007). The activity of fish AMPs has been tested not only against the more common fish bacterial pathogens (Noga, Silphaduang, Park, Seo, & Stephenson, 2009), but also against other pathogens like nervous necrosis virus (Chia, Wu, Chen, & Chi, 2010). Moreover, some AMPs have shown dual functional aspects, like hepcidins that have been indicated to be involved in iron regulation (Shi & Camus, 2006). Piscidins have been demonstrated to be present both in mast cells and professional phagocytic granulocytes (Mulero, Noga, Meseguer, Garcia-Ayala, & Mulero, 2008) and have been detected via bug blot, Western blot, ELISA, and/or immunochemistry in gill extract of different important fish species (Corrales, Mulero, Mulero, & Noga, 2010).

Due to their impact on the fish immune system, AMPs levels could be useful to determine, maintain, or improve fish health in aquaculture (Noga, Ullal, Corrales, & Fernandes, 2011). In fact, as an example, piscidin 2 estimated concentrations in different tissues of hybrid striped bass are lethal to different ectoparasites (Colorni, Ullal, Heinsch, & Noga, 2008) and piscidin 4 concentrations in gills are lethal to important bacterial pathogens (Corrales, Gordon, & Noga, 2009). Different chronic stresses lead to significant downregulation of AMPs and thus their monitoring could be useful in aquaculture to measure health status, and upregulation of AMPs could be of interest to enhance disease resistance and to improve the efficacy of traditional treatments against pathogens, like it happens for immunostimulants (Noga et al., 2011).

AMPs could be of great interest even in biomedicine, as they are attractive candidates for different therapeutic approaches. In mammals, AMPs have demonstrated diverse biologic effects (Guani-Guerra, Santos-Mendoza, Lugo-Reyes, & Teran, 2010), like enodotoxin neutralization, immunomodulating activity, and induction of angiogenesis and therefore they are seen as very attractive therapeutic tools. At the moment, the increasing incidence of antibiotic-resistant bacterial infections is of great importance in medicine and AMPs could help to cope with this challenge. They can combat different pathogens and exert their biological activity in several ways due to their multifunctional properties (Guani-Guerra et al., 2010).

Recently, the potential applications of fish trypsins in biomedicine have become relevant, though the features of fish trypsins from different species and their potential applications in different kinds of industries have been widely reported. The cold-adapted trypsin of Pacific cod (*Gadus macrocephalus*) has been proposed as an alternative to bovine trypsin in the synthesis of industrial peptides as its catalytic efficiency is 35 times greater than that of bovine trypsin (Fuchise et al., 2011). Both natural peptides and their synthetic analogues have interesting biological properties (stability, potency of action, and biological specificity) and are useful in different fields as therapeutic agents, synthetic analogues of peptide hormones, and in pharmacological applications, among others (Korhonen & Pihlanto, 2006).

A potential application for fish trypsins is as an antipathogenic agent. In vitro assays of the trypsin of Atlantic cod (*Gadus morhua*) has shown a high antipathogenic efficacy against HSV-1 and respiratory syncytial virus (RSV), the two most prevalent pathogenic viruses in upper respiratory tract infections. In addition, in vivo assays have shown favorable results using Atlantic cod trypsin in formulations to heal wounds.

The trypsin from Atlantic cod has been proposed for the development of cosmetics and medicines and six patents related to its production and use for the prevention and treatment of diseases have been registered. These enzymes inactivate bacterial enterotoxins, inflammatory cytokines, and cell-surface receptors involved in cell adhesion, and they have therapeutic use as a topical agent against pain, acute and chronic inflammation, rheumatic and autoimmune diseases, allergies, microbial infections, dermatopathies, and dental plaque remover (Bjarnason, 2000). In 2015 their use was extended as an adjuvant in the prevention and treatment of microbial infections in people with an immunodeficiency and patented as a novel treatment (Clarsund & Blom, 2015). In addition, novel trypsin isoforms called ZT have been described and patented (Gudmundsdottir & Scheving, 2017; Gudmundsdottir, Stefansson, & Scheving, 2015).

Recently, innovative strategies of trypsin administration have been studied to ensure that trypsins retain their properties until they exert their action. To achieve this, trypsin was nanoencapsulated with chitosan and supplied to rohu (*Labeo rohita*), which led to an increase in the efficiencies of productive fish compared to the control fed with unencapsulated trypsin. A similar administration in humans could contribute to the treatment of some health disorders related to the deficiency of pancreatic enzymes, and in animals, this administration strategy could facilitate the digestion of feeds based on vegetable proteins (Kumari et al., 2013).

The biochemical and kinetic properties of several native fish trypsins of different species have been reviewed by Bougatef (2013). In this context, the molecular weight of the enzymes reported was in the range of 22−30 kDa, while the optimum temperature was between 40°C and 65°C and the optimum pH was in the range of 8−11. The trypsins have esterase and amidase activity; in both cases, the mechanism involves the transfer of the acyl group to water. On their natural substrate, the trypsins catalyze the hydrolysis of peptide bonds at the carboxyl side of the lysine or arginine residues.

The optimal temperature of temperate-zone fish trypsins, such as those obtained from jacopever (*Sebastes schlegelii*), spotted mackerel (*Scomber australasicus*), true sardine (*Sardinops melanostictus*), yellow tail (*Seriola quinqueradiata*), and Japanese anchovy (*Engraulis japonicus*), is slightly higher (60°C), while the optimal temperature of the temperate-zone trypsins is similar to those of tropical fish trypsins from Nile tilapia (*Oreochromis niloticus*), Atlantic bonito (*Sarda sarda*), skipjack tuna (*Katsuwonus pelamis*), bluefish (*Pomatomus saltatrix*), tongol tuna (*Thunnus tonggol*), and yellowfin tuna (*Thunnus albacores*), which have an optimal temperature of 55°C−65°C (Kishimura, Tokuda, & Yabe, 2007; Klomklao et al., 2006; Klomklao, Benjakul, & Visessanguan, 2007).

Even though the optimal temperatures for cold-zone fish trypsins are high compared to the temperatures in which these organisms live (Kishimura, Klomklao, Benjakul, & Chun, 2008), their thermal stabilities are lower and, in general, are less than 40°C. Cold-adapted fishes have been found with some trypsin isoforms that work at high temperature and are classified within the mesophilic group.

In general, fish trypsins have an optimal pH in the range of 7−11 and are stable at alkaline pH (7−9), but their stability decreases in acidic pH (less than 6) (Bougatef et al., 2010; Khandagale, Mundodi, & Sarojini, 2017). However, some fish trypsins are stable over a wider pH range, such as the case of the Tunisian barbel (*Barbus callensis*) and striped seabream (*Lithognathus mormyrus*) trypsins that preserve above 90% of the enzymatic activity in a pH range of 5−12 (Elhadj-Ali, Hmidet, Bougatef, Nasri, & Nasri, 2009; Sila et al., 2012); the zebra blenny trypsin (*Salaria basiliscа*) that conserves 100% of the enzymatic activity in a pH range of 7.0−12.0; and the trypsins of pectoral rattail (*Coryphaenoides pectoralis*) and Bogue (*Boops boops*) that conserve above 70% of the enzymatic activity in a pH range of 6−11 (Barkia et al., 2010; Klomklao, Kishimura, & Benjakul, 2009; Ktari, Ben Khaled, & Nasri, 2012)—all of them are present in the Mediterranean sea.

Recently Luer and Walsh (2018) published a very interesting review about potential human health applications from marine biomedical research with elasmobranch fishes. Members of the subclass of fishes collectively known as elasmobranchs (Class: Chondrichthyes, Subclass: Elasmobranchii) include sharks, skates, rays, guitarfish, and sawfish. These fish species diverged from the main line of vertebrate evolution some 400 million years ago and continue to be successful in our ever-changing oceans. Much of their success must be attributed to their uncanny ability to remain healthy. Based on decades of basic research, some of their secrets may be very close to benefitting man.

For thousands of years, cultures around the world have envisioned the sea as a potential treasure for remedies to human ailments. The mystique surrounding medicinal secrets from the oceans has continued into modern times, with the quest to discover "drugs from the sea" (Malve, 2016). While a handful of drugs have been developed from marine invertebrates, marine vertebrates have remained underutilized as a potential source for new therapeutic agents. Sharks and their skate and ray relatives are collectively termed elasmobranchs (Class: Chondrichthyes, Subclass: Elasmobranchii), where much of their success during their evolution can be attributed to their numerous sensory systems (some of which are the most sensitive in the animal kingdom) (Hueter, Mann, Maruska, Sisneros, & Demski, 2004), their reproductive strategies (more similar to birds and mammals than to the bony fishes) (Carrier, Pratt, & Castro, 2004), and their uncanny ability to remain relatively disease-free. It is rare to find a "sick" shark in the wild, with the principal causes of death attributed to anthropogenic interaction (namely overfishing) (Davidson, Krawchuk, & Dulvy, 2016; Dulvy et al., 2014), predation (Heithaus, 2004), and natural senescence (old age) (Cailliet & Goldman, 2004).

Unless new and improved antibiotics are discovered, effective treatment of bacterial, fungal, parasitic, and viral infections, as well as chronic diseases, including cancer, will continue to be a challenging task for humans. Elasmobranchs might be a surprisingly rich source of novel antibiotics, if we consider the recurring observation of infection-free healing of wounds among them (Domeier & Nasby-Lucas, 2007; Towner, Smale, & Jewell, 2012). In 2017 it was possible to isolate 1860 bacterial symbionts from the epidermal mucus of three stingray and one skate species (Ritchie et al., 2017), which were screened for their abilities to produce antibacterial compounds with inhibitory activity against a range of pathogenic test strains; 311 (16.7%) of the isolates demonstrated activity against one or more of the pathogens, 57 of which produced either broad-spectrum antibiotics or activities against methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE) only.

Similarly to stingrays, sharks produce epidermal mucus, but because of the characteristic presence of superficial dermal denticles on shark skin, their mucus is not as accessible. As is often the case, there are exceptions, as two recent studies have demonstrated that mucus-associated bacteria from six species of shark possess antibiotic activity. In one study, antibiotic activity was detected in 41% of bacterial associates from blacktip sharks, *Carcharhinus limbatus*; 29% from tiger sharks, *Galeocerdo cuvier*; 13% from bull sharks, *Carcharhinus leucas*; 10% from lemon sharks, *Negaprion brevirostris*; and 7% from blacknose sharks, *Carcharhinus acronotus* (Ritchie et al., 2017). In the second study, as much as 20% of the culturable bacterial isolates from the mucus of white sharks, *Carcharodon carcharias*, was shown to produce antibacterial activity (Ritchie, Gil-Agudelo, Conrad, & Fisher, 2018), which is one of the most threatened shark species in the planet. Such a growing database of antibiotic-producing marine bacteria has implications for host−microbe associations among the elasmobranch fishes and may reveal promising candidates for future drug discovery initiatives.

The isolation and purification of an aminosterol from shark stomach tissue with broad-spectrum antifungal, antibacterial, and antiprotozoal activity was described in 1993 (Moore et al., 1993). This compound (a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3) was named squalamine, because it was isolated from the spiny dogfish (*Squalus acanthias*) (Fig. 5.2), which has become an important commercial fisheries target species in the Mediterranean recently. Squalamine can be synthesized (Zhang et al., 1998), though it is also present in other shark tissues including liver and gall bladder. This is a significant development in that the natural population of sharks will not be relied upon as a constant source of this product.

Squalamine has also been found to be antiangiogenic, a property useful in inhibiting the growth of solid tumors, because solid tumors depend upon the recruitment of blood vessels to thrive. Squalamine has been shown to have antitumor activity in rodent models of human brain, breast, lung, and ovarian cancers (Schiller & Bittner, 1999). Several phase I and phase II human trials using squalamine have been conducted (Bhargava et al., 2001, Hao et al., 2003; Herbst et al., 2003), although to date none of these studies have resulted in successful phase III trials. Squalamine does not appear to be related in chemical structure or mechanism of action to any currently known and used chemotherapeutic substance.

More recently, clinical tests have been initiated with squalamine in the form of squalamine lactate (Ciulla, Oliver, & Gast, 2007) to investigate its antiangiogenic properties to treat the eye disease known as age-related macular degeneration (AMD) (Mogi, Adams, Ji, & Mainolfi, 2013; Smith & Kaiser, 2014), a leading cause of blindness in older people. Future applications of squalamine may take advantage of another of its properties, namely antiviral activity. In fact, squalamine has been tested against a broad spectrum of human viral pathogens, including single positive-stranded RNA viruses associated with dengue, yellow fever, and equine encephalitis, and the double-stranded DNA hepatitis B virus (Zasloff et al., 2011). Squalamine, with its net positive charge by virtue of its spermidine moiety, displays a high affinity for anionic phospholipids. When it enters a cell, squalamine binds to the intracellular membrane phospholipids, neutralizing the negative charge and displacing any positively charged proteins bound to the membrane; this impacts the cell's ability to support virus replication. The role of squalamine in sharks, skates, and rays remains unclear, although it has been hypothesized that squalamine might also serve an antiviral function within the shark (Zasloff et al., 2011).

FIGURE 5.2 Photo of Squalus spp. individual fished in the Albanian territorial waters (photo provided by R. Bakiu).

Squalamine also shows tremendous promise with Parkinson's disease, a disease characterized by the presence in brain tissue of aggregates primarily formed by the protein α-synuclein (Perni et al., 2017). Squalamine suppresses the formation of α-synuclein aggregates and their associated toxicity in neuronal cells by competing with α-synuclein for binding to lipid membranes (Wang, Gerstein, & Snyder, 2009).

The transcriptome represents the complete set of genomic transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Cellular genetic information is transcribed into RNA, and the resulting readouts of all the genes of a given cell are referred to as its transcriptome.

While the human genome has been studied for many years, the first high-quality genome sequence generated from a cartilaginous elasmobranch relative was from the elephant shark, *Callorhinchus milii* (Class Chondrichthyes, Subclass Holocephali) (Venkatesh et al., 2014), followed closely thereafter by a complete mitochondrial genome of the great white shark, *C. carcharias* (Chang, Shao, Lin, Fang, & Ho, 2014). Subsequently, additional elasmobranch genome projects have been initiated (Wyffels et al., 2014), with transcriptomes now available for the whale shark, Rhincodon typus (Read et al., 2017); great white shark, *C. carcharias* (Richards, Suzuki, Stanhope, & Shivji, 2013); catshark, *Scyliorhinus canicula* (Mulley, Hargreaves, Hegarty, Heller, & Swain, 2014); spiny dogfish, *S. acanthias* (Chana-Munoz et al., 2017); and little skate, *Leucoraja erinacea* (Wang et al., 2012).

Recently, Hara and coworkers (2018) provided complete genome analyses of the brownbanded bamboo shark, *Chiloscyllium punctatum*, and cloudy catshark, *Scyliorhinus torazame*, plus an improved assembly of the whale shark genome that revealed important discoveries with regard to Hox genes, antibody genes, and opsin and olfactory receptor genes.

Genomics and transcriptomics studies provide a basis for molecular exploration of phenotypes unique to elasmobranchs, as well as insight into the evolutionary origins of vertebrates. Genomic analysis of their established traits of morphology, reproduction, sensory capabilities, and longevity, combined with their slow rate of DNA evolution (Martin, Naylor, & Palumbi, 1992), has the potential to elucidate specific molecular mechanisms underlying these unique features.

Elasmobranch transcriptomes may be more useful for human biomedical applications than initially thought. A recent comparison of gene transcripts between the greatwhite shark, *C. carcharias*, and zebrafish, *D. rerio*, revealed the surprising result that great white shark gene products associated with metabolism, molecular functions, and the cellular locations of these functions were more similar to human than to zebrafish (Zhen et al., 2015). In fact, these same shark transcriptome gene expression studies have identified positive selection for genes, such as legumain, that play important roles in immune system responses to certain cancers, including colorectal cancer (Zhen et al., 2015).

An interesting feature of the shark genome is the high proportion of dinucleotide microsatellite repeats, with a lower abundance than other vertebrates of repeating trinucleotide DNA sequences (Richards et al., 2013). This observation is worthy of note as abnormally higher numbers of trinucleotide repeats in humans have been linked to a variety of neurological disorders, including spinobulbar muscular atrophy, myotonic dystrophy, and Huntington's disease, as well as certain types of cancer (i.e., hereditary nonpolyposis colon carcinoma and sporadic bladder carcinoma) (Arzimanoglou & Barber, 1998; Panzer, Kuhl, & Caskey, 1995; Reddy & Housman, 1997). While it is difficult to assess neurological disease in elasmobranchs, the relatively lower proportion of trinucleotide microsatellite repeats in the great white shark genome may provide a genetic mechanism for the relatively low incidence of malignant neoplasia among elasmobranchs (Richards et al., 2013). As transcriptomes from more species become available, the transcriptome assemblies and the derived gene transcripts will be invaluable as new molecular tools in support of ongoing research with elasmobranch models (Marra et al., 2017).

5.4 Conclusions

Differently to the other marine organisms (bony fish, mollusks, and echinoderms), potential biomedical applications of elasmobranch research are starting to receive favorable attention with advances in the understanding of elasmobranch physiology, especially the immune system. Recent advances are leading to the development of new genomics tools, and the discovery of novel antimicrobials and antibody structures, as well as compounds produced by unique immune tissues of elasmobranch fishes. Anyway, it is important to know that the marine biodiversity should be conserved based on the facts that the generation of new tools and new approaches are exploring new biomedical potentials of the marine fish species (bony fish and elasmobranch fish species). The efforts toward greater insights should be combined with the efforts toward increasing the awareness of protecting the biodiversity of marine fish, which are revealing themselves to offer potential benefits to human health.

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Chapter 6

Superbugs, silver bullets, and new battlefields

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6.1 Introduction

The second half of the 20th century witnessed two major developments in the field of infectious diseases, the discovery of antibiotics and the emergence of resistance in pathogenic bacteria against antibiotics. Antibiotics are small molecules of microbial origin capable of, at lower concentrations, inhibiting growth or even destroying other bacteria selectively (Cavalieri, 2005). After their discovery, antibiotics were considered wonder drugs, a panacea for all infectious diseases. But it appears that these silver bullets are losing their effectiveness due to the emergence of superbugs or hard to treat bacteria. Superbugs are the bacterial pathogens capable of surviving in the presence of antibiotics and can cause diseases in patients. Although the dawn of the golden age of antibiotics saved many lives which couldn't be cured otherwise, often antibiotics are not any more working. But with the end of this golden age came the nightmare of antibiotic resistance and superbugs. There are many factors behind this failure. Most importantly many antibiotics are unable to inhibit the growth of bacterial pathogens because bacteria have modified themselves to avoid the severe effects of antibiotics.

Superbugs are capable of causing complicated and persistent nosocomial infections (Welsh, Bentz et al., 2017). Superbugs' share of hospital-acquired infections are increasing globally and are ranked among leading causes of morbidity (Aliyu, Smaldone, & Larson, 2017; Nithya, Gladstone et al., 2017; Spellberg, Powers, Brass, Miller, & Edwards, 2004). In a recent surveillance study by International Network for Optimal Resistance Monitoring (INFORM) about 10% of the isolated pathogens were classified as extensive drug-resistant microbes (Sader, Huband, Castanheira, & Flamm, 2017). In 2017 the World Health Organization expressed the need to develop/design antibacterial strategy against clinically prevalent superbugs. At the moment antibiotics are the only options used across the globe to treat patients with bacterial infections and if newer approaches and alternatives are not considered one might experience the mortality and morbidity of the preantibiotic era. The lack of vaccines against most of the nosocomial infectious agents leads to another critical situation for patients in intensive care units and/or those who are immunocompromised (Priebe, Meluleni, Coleman, Goldberg, & Pier, 2003). The major reasons behind the failure of these magic drugs are careless drug administration and treatment, the use of broad-spectrum antibiotics, that is, "one size fits all," and the alteration of cellular targets by bacteria. In this review we have discussed the current situation of the antibiotic resistance phenomenon which is gradually attaining a pandemic status, the factors responsible, and potential alternative options for tackling this situation.

6.2 Antibiotics and resistance

The three resistance mechanisms are alteration of cellular targets of antibiotic actions, enzymatic inactivation of antibiotics, and excretion of drug molecules via active transport drug efflux pumps. Superbugs use these mechanisms all the time to avoid the drastic effects of antimicrobial chemotherapeutic agents (Alpert, 2017; Nisbet, 2016; Nizet, 2015). This phenomenon has rendered many antibiotics ineffective as their target cellular sites were no longer the same as

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those that were previously susceptible (Sommer, Munck, Toft-Kehler, & Andersson, 2017). This problem has to be overcome by adopting new strategies for designing new antibiotic drugs. Using new targets will help the physicians in an arms race against superbugs (Munguia & Nizet, 2017). Using virulence inhibitors or disarming the pathogens is one of these new strategies that could help in overcoming this problem. These types of inhibitors stop the virulence factors produced by superbugs from causing damage to the host's body (Ruer, Pinotsis, Steadman, Waksman, & Remaut, 2015). These inhibitors do not place a selective pressure on the microbes, as conventional antibiotics do, but leave them unable to cause any harm to the body (Andersson & Levin, 1999; Levy & Marshall, 2004; Clatworthy, Pierson, & Hung, 2007). Thus this approach will be a good remedy for patients suffering from superbug infections.

The clinical and drug administration practices like overuse, misuse, and self-prescription are the key factors fueling the emergence of resistance in bacteria. Alexander Fleming in his Nobel lecture raised the fears of the emergence of resistance and even deaths due to superbugs due to careless treatment practices (Fleming, 2014; web. January 26, 2018). Fleming's fear about the loss of Penicillin's efficacy didn't take long to become a reality and now the deployment of any new antibiotic faces the emergence of resistance within a short time span (Clatworthy et al., 2007). So far clinical guidelines related to antibiotic therapies have been focused on optimum treatment efficacy with minimum degree of toxicity (Geli, Laxminarayan, Dunne, & Smith, 2012). But this approach has led to even greater selective pressure on microbes and thus in turn resulted in more resistance (Geli et al., 2012).

Over a period of time antibiotics have been used in agriculture and animal husbandry, not just for therapeutic and pesticide purposes, but also for increasing weight in farm animals. Though the European Union has banned this practice, it is still used in many different countries including the United States (Sneeringer, MacDonald, Key, McBride, & Mathews, 2017). There have been concerns about the possible correlation between antibiotic-fed animals and drug resistance in humans. This practice indicates a side effect of antibiotics on animal bodies, obesity. Although farmers are utilizing this aspect for obtaining animals with high meat yields at lower levels of antibiotics, this results in the incorporation of clinically important gut microbes into superbugs (Levy & Marshall, 2004). Moreover excessive amounts of antibiotics are also accumulating in the environment and affect normal microbial population, and thus ultimately affect the whole ecosystem indirectly with greater populations of superbugs in the environment.

The development of virulence inhibitors will be of greater clinical importance in the near future as these therapeutic agents will render superbugs disarmed. Disarmed microbes will be capable of growth but will never be able to cause diseases. Careless clinical and drug administration practices were the concerns raised by Alexander Fleming and which now have brought us ineffective antibiotics. Clinical guidelines need to be revised for the appropriate treatment of patients suffering from bacterial infectious diseases. By estimating effective drug dose and period of administration the resistance phenomenon could be handled properly. Along with the resistance management, the isolation and development of newer antibiotics are also needed.

Pseudomonas aeruginosa is one of most common cystic fibrotic nosocomial pathogens. It causes chronic nosocomial infections in hospitalized, immune-compromised, and transplant recipient patients. *P. aeruginosa* has a well-equipped arsenal of virulence factors and it tends to acquire drug resistance which helps in the establishment and progression of infection. Infections from MDR strains of *P. aeruginosa* in immune-compromised and transplant patients are becoming a very serious healthcare issue (Carmeli, Armstrong et al., 2016; Fernandes, Vira, Medikonda, & Kumar, 2016). Biofilm formation by *P. aeruginosa* is considered as one of many virulence factors, which help individual bacterial cells to survive antibacterial chemotherapy in a coordinated manner via quorum sensing (QS) (Winstanley & Fothergill, 2009). QS circuits in *P. aeruginosa* biofilms are complex and multicomponent interaction and regulatory systems regulate the production of various other virulence factors (Bodelón, Montes-García et al., 2016).

One of the key problems associated with the current healthcare situation is the phenomenon of increasing drug resistance and unresponsiveness. The clinical and drug administration practices like overuse, misuse, and self-prescription are the key factors fueling the emergence of resistance in bacteria, and now deployment of any new antibiotic faces the emergence of resistance within a short time span (Clatworthy et al., 2007). *P. aeruginosa* carries many resistance mechanisms (Chalhoub, Pletzer et al., 2017). Resistant strains possess nearly all of the drug resistance mechanisms found in the microbial community. Mobile genetic elements are the prime reason for the inter- and intraspecies spread of these drug resistance genes (Ventola, 2015). These include active efflux pumps, β-lactamases, enzymatic modification, and spontaneous mutational alteration of the drug target site. The simultaneous presence of different mechanisms of resistance is leading the development of multidrug resistance (Strateva & Yordanov, 2009).

The amide bond of the β-lactam ring is cleaved by penicilloyl-serine transferases or β-lactamases enzymes. The resultant product lacks antibacterial activity. *P. aeruginosa* produces a range of β-lactamases which confer resistance against β-lactam antibiotics. So far four classes of β-lactamases have been identified (A−D). Three of the four classes (A, C, and D) utilize serine-based mechanisms for cleaving the amide bond while class B requires zinc ions for their

activity and are known as metallo-β-lactamases (MBLs). Along with all these, *P. aeruginosa* is reported to have extended spectrum β-lactamases (ESBLs) as well (Strateva & Yordanov, 2009; Sykes & Matthew, 1976).

Active efflux of antibiotics, surfactants, and dyes is achieved by the production of many membrane-embedded drug efflux pumps. *P. aeruginosa* expresses both constitutively and transiently multiple drug efflux pumps. These pumps are capable of efficient transportation of antibacterial agents from cytoplasm to the environment, from where their reuptake is already hindered by membrane impermeability. Therefore these pumps maintain very low or negligible levels of antibiotics in cytoplasm. MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM are the well understood multidrug efflux pumps of *P. aeruginosa* (Aeschlimann, 2003; Enguéné, Yvette et al., 2015; Hasdemir, 2007; Li, Barré, & Poole, 2000; Livermore, 2001; Llanes, Hocquet et al., 2004; Yonehara, Yamashita, & Nakagawa, 2016).

Enzymatic modification of antibiotics is another resistance mechanism observed in *P. aeruginosa*. Three different antibiotic modifying enzymes (AMEs) aminoglycoside phosphoryltransferases (APHs), aminoglycoside adenylyltransferases (also known as nucleotidyltransferases) (AADs or ANTs), and aminoglycoside acetyltransferases (AACs) are reported in *P. aeruginosa*. These plasmid-encoded enzymes add the chemical moieties of phosphate, adenyl, or acetyl radicals to drug molecules. These structural alterations reduce the binding affinities of drug molecules for their target sites (Llano-Sotelo, Azucena, Kotra, Mobashery, & Chow, 2002; Ruppé, Woerther, & Barbier, 2015).

6.3 Drug resistance and tolerance

As discussed previously antimicrobial resistance is an ability to neutralize the effects of an antibiotic by preventing drug−target interaction utilizing multiple resistance mechanisms, while the tolerance is the capability of dormant cells to survive the bactericidal effects of antimicrobials without harboring any resistance mechanisms (Lewis, 2010; Lewis, 2012). This tolerance is conferred to the microbial population by the formation of persister cells. Dormant cells as persisters, constitute a small fraction of the total population in most of the bacterial species (Gardner, West, & Griffin, 2007). Persister cells remain in a latent or quiescent state. These were initially discovered by Joseph Bigger in 1944. Persisters are phenotypic variants of the population unlike the resistant cells which carry resistance genes.

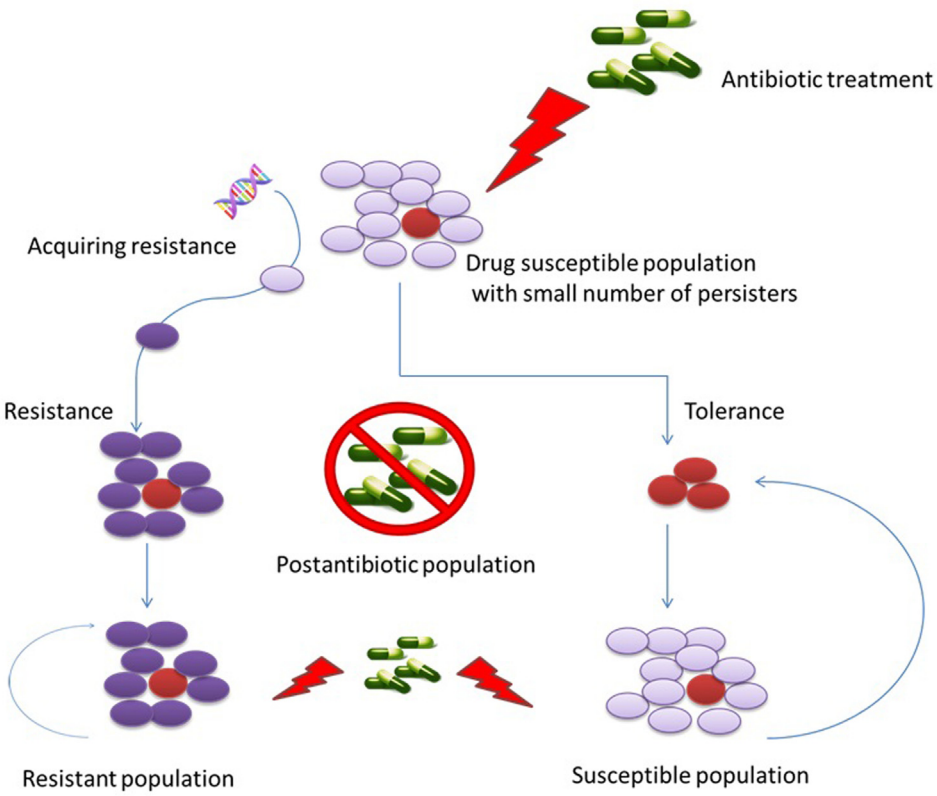

FIGURE 6.1 Schematic representation of drug resistance and tolerance in bacterial populations.

Fig. 6.1 represents the schematic depiction of resistance and tolerance phenomena in a bacterial population. Initially a bacterial population susceptible to antibiotics may or may not have resistance genotypes but persister phenotypes are generally reported in minute fractions. Upon exposure to an antibiotic the population dynamics begin to vary. The susceptible population is eliminated by the biocidal action of the antibiotic while the population undergoes selection to acquire resistance genes, whereas the persisters already present in the population in case of the unavailability of resistance mechanisms provide an alternative survival approach. Being dormant or nondividing, these cells are invulnerable to antibiotic activity. Both the resistance and tolerance mechanisms give rise to the subsequent population, but unlike resistant cells the consequent population that is produced by persisters is susceptible to future antibiotic treatments (Dawson, Intapa, & Jabra-Rizk, 2011; Lewis, 2012). Studies have reported the role of persisters in infection resilience (Conlon, Rowe, & Lewis, 2015).

6.4 Biofilms and resistance

Bacteria are capable of adopting and evolving their lifestyles depending upon environmental factors, that is, access to nutrients, temperature, salinity, pH, host-factors, and antibiotics etc. (Fux, Costerton, Stewart, & Stoodley, 2005). Changes in ideal growth conditions result in the implementation of survival strategies, including the formation of biofilm (Watnick & Kolter, 2000). Biofilms are complex multicellular associative structures where the members/inhabitants secrete extracellular polymeric substance (EPS) (Surette & Bassler, 1998) that are exhibited by various bacterial pathogens (Chmielewski & Frank, 2003; Gilbert, Khlebnikov, Cowan, & Keasling, 2001). Serving as fortresses, biofilms enhance the survival of the pathogen acting as an anvil receiving all the hammering (Kim & Kim, 2017). Biofilms are protective shields which hinder the diffusion of antibiotics due to the slimy nature of the overall structure, thus they act as nurseries for the emergence of multidrug-resistant organisms (Donlan, 2009). Studies have revealed the architectural complexities of biofilms (Watnick & Kolter, 2000; Wimpenny, Manz, & Szewzyk, 2000). Fig. 6.2 depicts the steps involved in the development of biofilm on a surface and the resistance mechanisms adopted as collective survival measures.

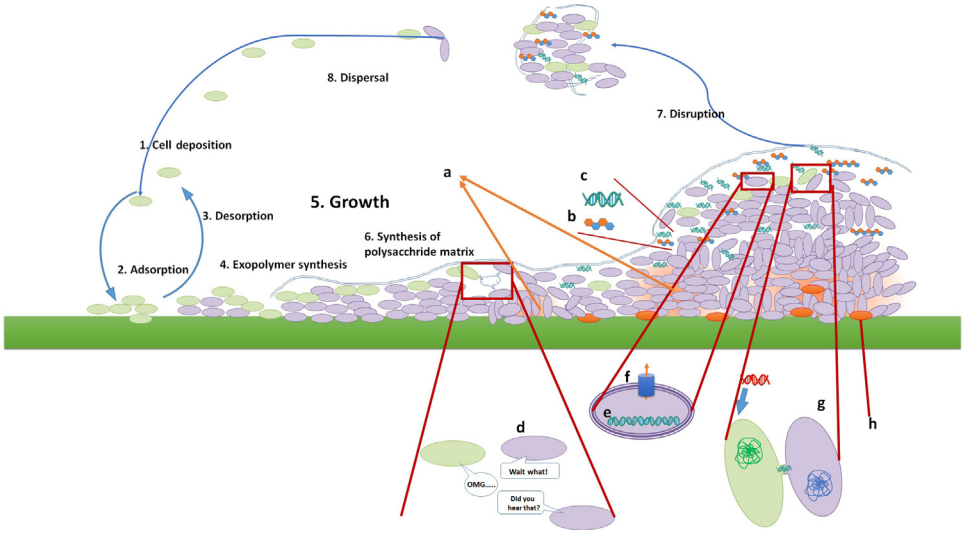

FIGURE 6.2 Schematic overview of biofilm formation and its architecture with employed resistance mechanisms. Biofilm formation begins: (1) with bacterial cells (light green and purple ovals) settling down on the surface from the aqueous environment and cell adhesion sets in action but (2) only reversibly attachment could be (3) disrupted at this stage due to environmental factors. (4) Gaining a foothold, irreversible adsorption on the surface bacteria proceeds toward secretion of exopolymer matrix. (5) As growth and expansion continues nutrient scarcity sets in distant innermost parts. (6) Synthesis of polysaccharide matrix in association with eDNA and proteins, yields an interactive scaffold which not only strengthens the overall architecture but also promotes cell–cell communication. (7) Excessive growth or physical impact reduces the overall architecture and results in detachment. Resistance mechanisms employed in biofilms: (a) Growth and metabolism result in creation of nutrient gradient with reduced nutrient supply at the core of architecture (b) exopolysaccharides in matrix (shown in yellow); (c) eDNA; (d) cellular signaling (quorum sensing, multispecies communication, etc.); (e) biofilm-specific translational profile; (f) multidrug efflux pumps; (g) horizontal gene transfer; and (h) persister cells.

6.4.1 Biofilm and superbugs

It has been long known that a wide range of bacterial species are capable of forming biofilms on both biotic and abiotic surfaces alike (Balzer & McLean, 2002; Costerton, Geesey, & Cheng, 1978; Sekhar, Kumar, & Chakraborti, 2009). Biofilms are of critical importance from the public health point of view since such associative structures provide the shields enabling the pathogens to fend off not only the antibacterial chemotherapeutics but also the host's immune system (Alhede, Bjarnsholt, Givskov, & Alhede, 2014; Roilides, Simitsopoulou, Katragkou, & Walsh, 2015), thus augmenting the survival of the pathogen and the progression of infection. All of the clinically significant bacteria are capable of forming biofilms coupled with antibiotic resistance (Hall & Mah, 2017). Biofilms can develop on prosthetics (Gries & Kielian, 2017), implants (Hahnel, Wieser, Lang, & Rosentritt, 2015; Mandell, Deslouches et al., 2017), and other internal medical, surgical, and miscellaneous devices (Götting, Klassen et al., 2016; Percival, Suleman, Vuotto, & Donelli, 2015; Zumstein, Betschart et al., 2017). Formation of complicated "pod like structures" with enhanced survival have been reported in animal model studies for *Escherichia coli* bladder infections (Cho, 2006; Naves, Del Prado et al., 2010). Upregulation of the genes (aaeX, yceP, ygiD, and yqgA) associated with biofilm formation have been reported in knockout studies, suggesting the overlapping of the expression profile with biofilm formation profiles of some *E. coli* strains (Hancock & Klemm, 2007). Studies in *P. aeruginosa* have also reported the roles of Chaperone-usher pathway (Cup) components in fibril-mediated bacterial adhesion and biofilm formation (Vallet, Olson, Lory, Lazdunski, & Filloux, 2001). Upon induction such genes result in excessive biofilm formation. The *cup* gene clusters act synergistically yet independently, in cell surface fimbriae assembly for achieving microcolony formation (Ruer, Stender, Filloux, & de Bentzmann, 2007).

The extent of diffusion of the antibiotic differs depending upon multiple factors, that is, bacterial strain used, experimental design, and culture growth conditions (Hall & Mah, 2017). Studies have shown variable degrees of penetration of different antibiotics against a range of pathogens with a spectrum of results: complete penetration without affecting cellular viability (Stone, Wood, Dixon, Keyhan, & Matin, 2002), effectively permeable at higher planktonic MICs (Anderl, Franklin, & Stewart, 2000; Boudjemaa, Briandet et al., 2016; Zahller & Stewart, 2002), and limited diffusibility (Singh, Ray, Das, & Sharma, 2010; Singh, Sahore, Kaur, Rani, & Ray, 2016). The nutritional composition of the culture media also affected drug penetration in an indirect manner (Suci, Mittelman, Yu, & Geesey, 1994; Tseng, Zhang et al., 2013; Vrany, Stewart, & Suci, 1997).

Extracellular DNA (eDNA) is among many structural and functional components of the bacterial biofilm matrix. The origins of eDNA could be bacterial or derived from host leukocytes (Allesen-Holm, Barken et al., 2006; Jakubovics, Shields, Rajarajan, & Burgess, 2013). Irrespective of its origin, eDNA fortifies the biofilms against some antibacterial agents (Chiang, Nilsson et al., 2013). The actual role of eDNA in contributing toward biofilm resistance is not clear, although studies have explored multiple mechanisms. The extracellular matrix environment can be altered by chelating cations, that is, magnesium, thus leading to the reduction of Mg^{2+} in the matrix (Mulcahy, Charron-Mazenod, & Lewenza, 2008). The formation of localized acidic microdomains has also been observed due to eDNA accumulation (Wilton, Charron-Mazenod, Moore, & Lewenza, 2016). Such environmental changes result in the alteration of the overall gene expression profile resulting in a cascade of transcriptional effects leading to the upregulation of the PA3552−3559 operon (McPhee, Bains et al., 2006). The resultant translational products perform aminoarabinose addition to the lipid A moiety of LPS, thus conferring resistance against aminoglycoside and cationic antimicrobial peptides (Lewenza, 2013).

Studies have revealed a higher degree of expression of chromosomal Pf phage genes in *P. aeruginosa* biofilms (Whiteley, Bangera et al., 2001). *P. aeruginosa* uses the filamentous phage particles for the organization of the biofilm in an orderly fashion to form a liquid crystalline matrix (Secor, Sweere et al., 2015). Phage particles trigger cell death, allowing the release of eDNA and the formation of drug-tolerant colony variants in the biofilm (Petrova & Sauer, 2011; Rice, Tan et al., 2009; Webb, Lau, & Kjelleberg, 2004; Webb, Thompson et al., 2003). The phage filaments are reported to filter out the biofilm matrix by binding with tobramycin due to surface charge and binding affinity (Secor et al., 2015).

Biofilms harbor ideal situations for horizontal gene transfer, that is, conjugation (Antonova & Hammer, 2011; Ghigo, 2001; Madsen, Burmølle, Hansen, & Sørensen, 2012). The presence of the conjugative F plasmid is reported to reduce the lytic bacteriophage infections. λ and T7 failed to penetrate due to the formation of exopolymers (Doolittle, Cooney, & Caldwell, 1995; May, Tsuruta, & Okabe, 2011). F pili provide the necessary cell-to-cell contact for biofilm formation (May et al., 2011).

A comparative study revealed the effect of oxygen on biofilm development to some extent, yet other factors, that is, the strain's genetic makeup, also influence the process (Bjergbæk, Haagensen, Reisner, Molin, & Roslev, 2006). Another study revealed the antibiofilm potential of human serum albumin (HSA), when tested against seven biofilm-forming *E. coli* strains (Naves et al., 2010).

6.4.2 Biofilm on indwelling medical devices

Most bacterial pathogens produce biofilm on a wide range of surfaces (Sekhar, Ohri, & Chakraborti, 2010). The majority of these biofilms are formed on medical indwelling surfaces, that is, contact lenses (Jain, Bhosale, & Tale, 2016; Konstantinović, Ćirković, Đukić, Marić, & Božić, 2017), catheters (Ren, Colletta et al., 2015), cosmetic and orthopedic implants (Costerton, Montanaro, & Arciola, 2005; Hu, Johani et al., 2016; Schierholz & Beuth, 2001; Williams, Sinclair, Jeyapalina, & Bloebaum, 2013), prosthetics (Gbejuade, Lovering, & Webb, 2015; Gomez, Cazanave et al., 2012), devices (Abdel-Hafeez, El-Mehallaway, Khalil, Abdallah, & Elnaggar, 2014; Mounier, Kapandji et al., 2016; Wu, Moser, Wang, Høiby, & Song, 2015), and surgical equipment (Bayston, Ashraf et al., 2007; Elgharably, Mann et al., 2013; Garrett, Bhakoo, & Zhang, 2008; Murdoch, Taylor et al., 2006). The development of biomaterials capable of resisting biofilm formation has been in consideration for a while now (Campoccia, Montanaro, & Arciola, 2013). These approaches involve surface coating with antibacterial nanoparticles and biomaterials (Carlson, Taffs, Davison, & Stewart, 2008; Hetrick, Shin, Paul, & Schoenfisch, 2009; Qin, Cao et al., 2014). The manufacturing of biomedical devices and scaffolds and other tissue engineering materials relating to biomaterial is globally US$170 billion per annum and growing (Bryers & Ratner, 2004).

6.5 Spread of Resistance

6.5.1 Tools and Mechanisms

Studies have reported the presence of antibiotic-resistant genes in microorganisms physically, geographically, and ecologically separated from modern human antibiotic use (Bhullar, Waglechner et al., 2012; D'Costa, King et al., 2011; Hernández, Stedt et al., 2012).

Since the origins of most antibiotics are traced back to microbes, evolutionarily the protective mechanisms against these antibacterial agents also emerged as a consequence of exposure. Competition over habitat and resources in a multispecies microbial community drove the development of such bacteriocidal and bacteriostatic agents, favoring by natural selection the emergence of resistance genes/mechanisms. Yet the pandemic extent of the situation faced today is the outcome of improper drug administration practices, as predicted by Fleming himself (Decousser, Poirel, & Nordmann, 2001; Fleming, 2014; Humeniuk, Arlet et al., 2002; Wellington, Boxall et al., 2013).

During the course of evolution bacteria have developed sophisticated mechanisms for lateral gene transfer, including conjugation, transduction, and transformation, and are also capable of utilizing mobile genetic elements (i.e., bacteriophages, genomic islands Plasmids, or transposons) for the exchange of information (Frost, Leplae, Summers, & Toussaint, 2005; Goldenfeld & Woese, 2007; Koonin & Wolf, 2008). The acquisition of more than two-thirds of the total genes during this evolution is attributed to horizontal gene transfer (Kloesges, Popa, Martin, & Dagan, 2010; Popa & Dagan, 2011). The abundance of horizontal gene transfer events and their role in microbial evolution is clearly associated with the competitive edge attained by microbes (Frost et al., 2005; Goldenfeld & Woese, 2007; Juhas, van der Meer et al., 2009).

Transformation is a complex process in which DNA is transported from the environment into the cell across the cell membrane. The uptake of DNA requires multiple transmembrane protein transport complexes, that is, type II secretion systems (T2SS), type IV secretion systems (T4SS), and type IV pili (Krüger & Stingl, 2011). Recent studies by Krüger & Stingl (2011) and Stingl, Müller, Scheidgen-Kleyboldt, Clausen, & Maier (2010) have revealed the mechanism of DNA transport into the cytoplasm to be a two-step process in which DNA is initially moved from the surface to the plasma membrane and then later into the cytoplasm (Krüger & Stingl, 2011; Stingl, Müller et al., 2010). To avoid the uptake of potentially hazardous DNA (naked phage genome or any other lethal/suicidal gene), this two-step mechanism might also be providing some resistance. Some microbial species like *Neisseria* and *Haemophilus* deal with this problem by restricting the import of DNA by sequence specificity, thus recognizing sequences capable of recombination with the cellular genome (Ambur, Davidsen et al., 2009; Ambur, Frye, & Tønjum, 2007; Treangen, Ambur, Tonjum, & Rocha, 2008).

Transduction or the transfer of genes via bacteriophages is a significant lateral gene transfer mechanism. Phages are considered as efficient gene transfer tools. They have been reported to transfer virulence and resistance genes in the vast majority of pathogenic and nonpathogenic microbes, such as *Staphylococcus aureus*, *P. aeruginosa*, *Bacillus subtilis*, *Rhodobacter capsulatus*, *Mycobacterium tuberculosis*, *E. coli,* and *Salmonella enterica* (Bibb, 2004; Canchaya, Fournous, Chibani-Chennoufi, Dillmann, & Brüssow, 2003; Coleman, Sullivan et al., 2006; Lang & Beatty, 2000;

Nakayama, Takashima et al., 2000; Novick, Christie, & Penadés, 2010; Ruzin, Lindsay, & Novick, 2001; Scott, Thompson-Mayberry, Lahmamsi, King, & McShan, 2008).

Horizontal gene transfer via conjugation or cell-to-cell exchange of DNA employs T4SS, which transports single-stranded DNA segments from donor to recipient cells (Alvarez-Martinez & Christie, 2009; Harms, Segers et al., 2017; Hughes & Andersson, 2017; Juhas, Crook, & Hood, 2008). The typical T4SS conjugation systems include T4SS (transferosome) complex, DNA binding complex (relaxosome), and linker protein connecting the two complexes (Dostál, Shao, & Schildbach, 2010; Wong, Lu, & Glover, 2012). Recently identified T4SS subfamily complexes in *Haemophilusas* and other species are reported to be responsible for the spread of drug resistance and virulence determinants and other genomic islands (Juhas et al., 2008; Juhas et al., 2009; Juhas, Crook et al., 2007). Studies have reported a novel intercellular communication system composed of intercellular nanotubes transporting proteins, metabolites, mRNA, and plasmids between adjacent cells in *B. subtilis* (Dubey & Ben-Yehuda, 2011). These nanotubes or "microbial superhighways" were observed between Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacterial species. The exact mechanism of production of these nanotubes is not completely understood and needs deeper insight (Dubey & Ben-Yehuda, 2011; Ficht, 2011; Schertzer & Whiteley, 2011).

Transposons require transposase for insertion and excision into the host genome. This insertion and excision is conducted in a sequence-specific manner and requires specific insertion sequences (IS) (Juhas et al., 2009; Roberts, Chandler et al., 2008). Integrative and conjugative elements (ICEs) are self-transmissible mobile genetic elements (Wozniak & Waldor, 2010). ICEs are capable of integration into the host genome and replicate along with the host genome and could also be transported via conjugation, thus sharing features from all the other mobile genetic elements, that is, phages, plasmids, and transposons (Gogarten & Townsend, 2005; Nakayama, Yamashita et al., 2008; Whittle, Shoemaker, & Salyers, 2002). ICEs were initially reported in *Enterococcus faecalis in* (Tn916) in 1980 and *Bacteroides thetaiotaomicronin* (CTnDOT) in 1988 (Scott & Churchward, 1995; Whittle et al., 2002). Since then multiple families have been reported in a wide range of bacterial species (Burrus, Marrero, & Waldor, 2006; Rodríguez-Blanco, Lemos, & Osorio, 2012; Song, Yu et al., 2013; Taviani, Ceccarelli et al., 2008; Wozniak, Fouts et al., 2009).

Microbial pathogens are enjoying an enormous drug resistome—the collection of genes conferring drug resistance—against our increasingly ineffective drug arsenal (Zhaxybayeva & Doolittle, 2011). This resistome is widely distributed between both pathogens and nonpathogens. This spread has been accelerated by careless usage of antibiotics.

6.6 Bacterial social interactions' influence on drug resistance

6.6.1 Bacterial persistence

Bacterial populations harbor small groups of cells invulnerable to catastrophic events such as antibiotic treatment, known as them from resistant mutants (Lewis, 2012). Persister cells remain in a quiescent or slow-growing state and do not exceed >1% of the total population (Gardner et al., 2007). This highest ratio is achieved during a stationary phase or biofilms. Without harboring any genetic diversity persisters are able to survive antibiotic treatment, and result in the regeneration of bacterial populations with the same degree of susceptibility, indicating that persisters-mediated survival is dependent on genetic drug resistance. With the greatest ratio in biofilms, presisters present a serious challenge to treatment of bacterial infections, especially in scenarios of nosocomial and other drug-resistant bacterial pathogens, that is, *M. tuberculosis*, *E. coli*, *S. enterica*, *P. aeruginosa*, and *S. aureus* (Pearl, Gabay, Kishony, Oppenheim, & Balaban, 2008; Dawson, Intapa et al., 2011). Persistence is not limited to bacterial populations as persisters are discovered in *Candida albicans* biofilm as well (LaFleur, Kumamoto, & Lewis, 2006). Persisters act indirectly as an insurance policy for the whole population while reducing competition for related bacterial lines in a direct manner (Gardner et al., 2007). Persisters are produced by two different mechanisms: (1) type I persisters emerge during the stationary state and may require a starvation signal (Korch, Henderson, & Hill, 2003); while (2) the generation of type II persisters does not require any such signal and these are observed during the exponential state as well (Pearl et al., 2008).

Biofilms are responsible for the majority of human infections, while antibiotic tolerance is vital for biofilm survival (Keren, Shah, Spoering, Kaldalu, & Lewis, 2004). Persisters play their due role in providing drug tolerance in biofilms (Mulcahy, Burns, Lory, & Lewis, 2010). As mentioned, nosocomial infectious agents *E. coli*, *P. aeruginosa*, and *S. aureus* do not only form biofilms and exhibit persistence but often carry multiple drug resistances as well (Accogli, Giani et al., 2014; Aliberti & Kaye, 2013; Jamal, Rosenblatt et al., 2014; Spoering & Lewis, 2001). For the emergence of drug resistance in microbes the growth dynamics could be different at the site of infection in a heterogeneous microbial population where a fraction of cells carry drug resistance along with the drug susceptible majority and persisters. Upon exposure to antibiotic stress the three groups will behave accordingly. A susceptible population will be eradicated

while reducing no competition for the resistant mutants and dormant persisters. In such condition the persisters are neither providing direct nor indirect benefits to the whole population, that is, reduced competition and survival.

E. coli is considered as a model organism for bacteria persistence. Studies on *E. coli*'s high-persister (hip) mutants have revealed much of the information regarding the induction of persistence and resuscitation in *E. coli*. Studies of hip mutants have revealed that this "noninherited" phenomenon is controlled by the hipBA operon (Korch & Hill, 2006; Korch et al., 2003). The hipBA operon is considered to be a type II toxin−antitoxin locus encoding two products toxin (hipA), which inhibits growth and induces persistence, and an antitoxin (hipB), which neutralizes the toxin's function (Bokinsky, Baidoo et al., 2013; Germain, Castro-Roa, Zenkin, & Gerdes, 2013; Han, Lee et al., 2010).

6.6.2 Bacterial population dynamics

Pathogenicity or virulence of bacterial communities is generally based on mutual cooperation among members of the community (Raymond, West, Griffin, & Bonsall, 2012). Bacteria not only inflict damage to their hosts by producing extracellular virulence factors, but also perform various vital tasks via these extracellular agents. These may include immune evasion, nutrient acquisition, quorum sensing, and biofilm formation (Brown, 1999; Diggle, Griffin, Campbell, & West, 2007; West & Buckling, 2003). Since these factors are poured into the extracellular environment the resultant benefits are shared with the neighboring cells (Sachs, Mueller, Wilcox, & Bull, 2004). The producer cells pay an extra metabolic cost for the production of these factors. This communal cooperation is susceptible to "cheating" by social cheats, the cells which do not produce such factors and take advantage of this communal cooperation (Frank, 1998; Nowak, 2006). This cheating behavior provides the cheaters with a "metabolic cost waiver" in the competition within the community (Brown, 2006). This phenomenon is also true for pathogenicity and virulence caused by extracellular virulence agents produced at the site of infection. In the case of the dependence of virulence on cooperation, the pathogenicity and epidemiology of the pathogen will be affected by the competition between the producers and cheaters present in the microcommunity (Brown, Hochberg, & Grenfell, 2002; Buckling & Brockhurst, 2008). This communal cooperation hypothesis has been evaluated in theory and in its practical aspects (Raymond et al., 2012; Strassmann, Zhu, & Queller, 2000). In a study Raymond et al. assessed the natural population dynamics of *Bacillus thuringiensis*. *B. thuringiensis* is used as a biocontrol agent against agricultural pests (Entwistle, 1993; Raymond, Johnston, Nielsen-LeRoux, Lereclus, & Crickmore, 2010). The experimental design focused on the evaluation of cooperation consequences at individual as well as population levels in a natural host−pathogen system of *B. thuringiensis* and the larvae of *Plutella xylostella*. The authors demonstrated the persistence of communal cooperation associated with virulence in the natural population, while social cheats do not account for significant disease outbreak (Raymond et al., 2012). Similar studies regarding quorum sensing, nutrient acquisition, and biofilm formation have been performed (Buckling, Harrison et al., 2007; Griffin, West, & Buckling, 2004; Nadell, Xavier, Levin, & Foster, 2008; Popat, Crusz et al., 2012; West, Diggle, Buckling, Gardner, & Griffin, 2007). This social equilibrium of producers and cheaters, if disturbed, in favor of cheaters during infection progression can lead to a reduction in virulence at the site of infection.

6.7 Alternative therapeutic approaches

6.7.1 Identification of new targets

Antibiotic targets in microbial cells are associated with cellular growth and multiplication, that is, cell wall synthesis, DNA replication, and protein translation (Goldberg, 1965; Hobby, Meyer, & Chaffee, 1942, Retsema, Girard et al., 1987, Smith, 1986). Disruption of these vital cellular processes results in an exertion of selection pressures on microbial populations to evolve and become either resistant or at least tolerant to these biocidal agents (Albrich, Monnet, & Harbarth, 2004; Allen, Donato et al., 2010; Ciusa, Furi et al., 2012; Kolář, Urbánek, & Látal, 2001; Tello, Austin, & Telfer, 2012). This situation demands the identification of novel drug targets which could be targeted by therapeutic agents without exerting selection pressure on microbes (Maura, Ballok, & Rahme, 2016; Rasko & Sperandio, 2010). Two key aspects covering our ultimate interest in superbug infections are virulence and resistance mechanisms; the mechanisms by which these notorious pathogens inflict damage to their hosts and manage to escape therapeutic agents. Designing alternative therapeutics focusing on virulence mechanisms could help in infection management while effectively circumventing the antibiotic-induced evolutionary selective pressure (Rampioni, Visca, Leoni, & Imperi, 2017). Disarming the superbugs would not only improve disease management and patient care but would also help host the immune system to easily deal with a comparatively vulnerable target (Chorell, Uvell, Pinkner, Hultgren, & Almqvist, 2014; Dickey, Cheung, & Otto, 2017; Heras, Scanlon, & Martin, 2015; Shoham & Greenberg, 2017).

Drug−drug interactions and chemical−protein interactions have long been used for the prediction of possible side effects of drugs. Yet these "side effects" may potentially hold the key for another clinical challenge (Luo, Zhang et al., 2014). Pyocyanin is considered as one of the primary virulence factors produced by *P. aeruginosa* with redox active activity (Lau, Hassett, Ran, & Kong, 2004). Drug repurposing studies have demonstrated antipyocyanin activity from a variety of drugs ranging from salicylic acid to raloxifene and their derivatives (Prithiviraj, Bais et al., 2005; Sui, Lo et al., 2012). Salicylic acid and its derivatives share structural similarity with the pyocyanin biosynthetic pathway precursor chorismic acid, and thus inhibit the pathways at early stages via competitive inhibition. Raloxifene is a selective estrogen receptor inhibitor used as an anticancer agent to avoid osteoporosis and invasive breast cancer (Gizzo, Saccardi et al., 2013). Raloxifene interacts with phenazine biosynthetic enzyme, PhzB2, in a dose-dependent manner and hinders pyocyanin production (Sui et al., 2012).

Baquero, Coque, & de la Cruz, 2011 have reviewed all such possible drug targets and targeting approaches (Baquero, Coque et al., 2011). In this review the authors have focused on "eco−evo strategies" (ecology and evolution) for drug targets, which could prevent, if not eliminate, the emergence and spread of drug resistance. The eco−evo concept could be explained as a study of organisms in light of their ecologic and evolutionary parameters rather than focusing on an organism's behavior as an infectious agent in laboratory settings or in the clinical practice (Dethlefsen, McFall-Ngai, & Relman, 2007; Pallen & Wren, 2007). Currently we are facing a huge influx of biological data regarding microbial population genetics, antibiotic resistance determinants, mobile genetic elements (MGEs), plasmids, transposons, and integrative conjugative elements (ICEs) (Carattoli, 2009; Frost et al., 2005; Skippington & Ragan, 2011; Smillie, Garcillán-Barcia, Francia, Rocha, & de la Cruz, 2010; Suzuki, Yano, Brown, & Top, 2010; Wozniak & Waldor, 2010). In the case of antibiotic treatment resistant genotypes are not only selected from the heterogeneous populations but the transfer and exchange of these resistance genes also take place via the abovementioned genetic transport vehicles (Couce & Blázquez, 2009; Courvalin, 2008; Prudhomme, Attaiech, Sanchez, Martin, & Claverys, 2006). This antibiotic-induced selection alters the ecological context, thus directing the evolution of microbial systems under antibiotic treatment (Baquero, 2009; Brown, West, Diggle, & Griffin, 2009). Since these genetic transport vehicles play their due role in this directed evolution (Baquero, 2009), these could become intervention targets against resistance (Baquero et al., 2011). The list of possible evo−eco targets discussed by Baquero et al. (2011) is shown in Table 6.1.

6.7.2 Bacteriophage cocktails

Bacteriophages are the viruses which prey on prokaryotes (Hermoso, García, & García, 2007; Roach & Donovan, 2015). Phages were discovered early in the 20th century by Felix d'Herrele (Goodridge & Abedon, 2003; Housby & Mann, 2009). Frederick Twort was the first person to discuss their antibacterial potential (Rohwer & Segall, 2015). The antibacterial activity of the phages had been observed even before their discovery as early as 1896 against *Vibrio cholerae* (Nigam, Gupta, & Sharma, 2014) and in 1898 against *B. subtilis* (Fard, 2016).

Phage therapy is the application of specific bacteriophages against respective bacterial pathogens to achieve bactericidal effects (Dubey, Chandraker, Sao, Gupta, & Dubey, 2016). The bacteriophage life cycle consists of two stages: the lytic cycle and the lysogenic cycle. Employing the phages' lytic life cycle as an antibacterial therapeutic phenomenon is shown in Fig. 6.3. Phages infect their respective host cells, multiply, and finally cause lysis of the cell. The released progeny phages continue to repeat this infectious cycle until either the host cells develop resistance against the phage or the phage switches to lysogenic mode (Ahiwale, Koparde, Deore, Gunale, & Kapadnis, 2012; Labrie, Samson, & Moineau, 2010; Wei, Ocampo, & Levin, 2010). Administration of phages could be done by oral ingestion for gastrointestinal tract infections, dermally for skin and wound infections, or by direct injection into tissues. But intravenous or systemic administration results in immune response and consequent phage inactivation (Westwater, Kasman et al., 2003).

Antibacterial activity has been studied for phages in animal models for evaluating their potential therapeutic applications (Kutateladze & Adamia, 2010; Smith & Huggins, 1982). An attempt to treat systematic and local infections of *Vibrio vulnificus* in mice by Cerveny, DePaola, Duckworth, & Gulig (2002) involved two lytic bacteriophages (CK-2 and 153A-5). Upon administration of phages (108 viruses/mice) resulted in significant reduction of pathogens (Cerveny, DePaola et al., 2002; Thompson, Iida, & Swings, 2004). In another phage therapy study for treating *V. cholerae*-phage coinfection experiments in murine models showed that phages have a negative impact on *V. cholerae* colonization (Nelson, Loomis, & Fischetti, 2001). However bacteria became more infectious at low *cfu* while facing a higher dose of phage. The mechanism of this phenotype is unclear and needs further studies. Bacteria might escape the lytic activity of phages, in coinfection experiments, by mutating their LPS surface markers. But mutation in LPS

TABLE 6.1 Possible eco–evo inhibitors of MDR bacteria and modes of action and effects (Baquero 2004).

General target	Intervention	Specific target	Example(s) of inhibitors
Penetration inhibitors	(i) Inhibitors of high-risk bacterial clones	Colonization factors	Synthetic multivalent glycoconjugates
		Surface antigens	Oral-mucosal vaccines. *Streptococcus pneumoniae* PCV7 and PCV13
		Quorum sensing (QS)	Anthranilic acid analogues, acylated homoserine lactones (AHLs), marine water-derived QS inhibitors (fimbrolides, manoalides, sesterterpenoids), analogs of acyl-homoserine lactones (AHLs), c tetramic and tetronic acids, halogenated furanones
		Regulators of colonization factor	Rho GTPase-activating bacterial toxins
	(ii) Reduction of selective environments for high-risk resistant clones	Antibiotics in upper gastrointestinal (GI) track	Beta-lactam agents, or antibiotic-binding substances
Promiscuity inhibitors	(i) Broad-host-range conjugation inhibitors.	Type IV secretion systems	Unsaturated fatty acids (e.g., dehydrocrepenynic acid, linoleic and linolenic acids)
			Thiadiazolidine-3,5-diones (compounds belonging to the Chiron Corporationsubstance libraries)
			Specific biphosphonates clodronate (Bonefos) and etidronate (Didronel)
			Salicylidene acylhydrazide (structurally similar to a class of molecules that have broad-spectrum activity against type III secretion T3S) systems
Plasticity inhibitors	(ii) Specific conjugation inhibitors.	Type IVB pili	Aptamer single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA)
	(i) Recombinase inhibitors	RecA	2-Amino-4,6-diarylpyridine compounds
	(ii) Mutation inhibitors	Integrase LexA (SOS gene networks)	Putative integrase inhibitors (as in HIV) Bacteriophages expressing uncleavable LexA variants (e.g., engineered M13 phage withlexA3 repressor)
		SoxRS (non-SOS-gene networks)	Bacteriophages overexpressing SoxRS
Persistence inhibitors	(i) Decontamination of high-risk clones.	Anticlonal vaccines	Streptococcus pneumoniaePCV7 and PCV13
		Essential genes for growth or virulence	Antisense oligomers peptide-conjugated PMOs (PPMOs), cationic PMOs (Gux-PMOs, Pip-PMOs) (PMOs are phosphorodiamidate morpholino oligonucleotides)
		Clonal interference strategies	Genetically modified organisms (GMOs), probiotics
	(ii) Decontamination of high-risk MGEs	Plasmid decontamination	Phenothiazines (such as promethiazine), dibenzoazepines, dibenzocykloheptene, plumbagin
		Exploiting plasmid incompatibility mechanisms (replication machinery)	Different inhibitors and approaches validated only in the laboratory
		Exploiting toxin-antitoxin (TA) systems	Homologs of thehokplasmid mantenance factor. Biological containment systems. GMOs carrying TA homologs
	(iii) Decontamination of high-risk genes	Antibiotic resistance genes	Antisense oligomers, such as phosphorothioate oligonucleotides (PS-ODNs), locked nucleic acids (LNAs), 2-O-methyloligoribonucleotides (2¢OMes), phosphorodiamidate morpholino oligonucleotides (PMOs), and peptide nucleic acids (PNAs)
		Antibiotic resistance genes, metabolic genes	Short double-stranded RNAs (small interfering RNA siRNA)

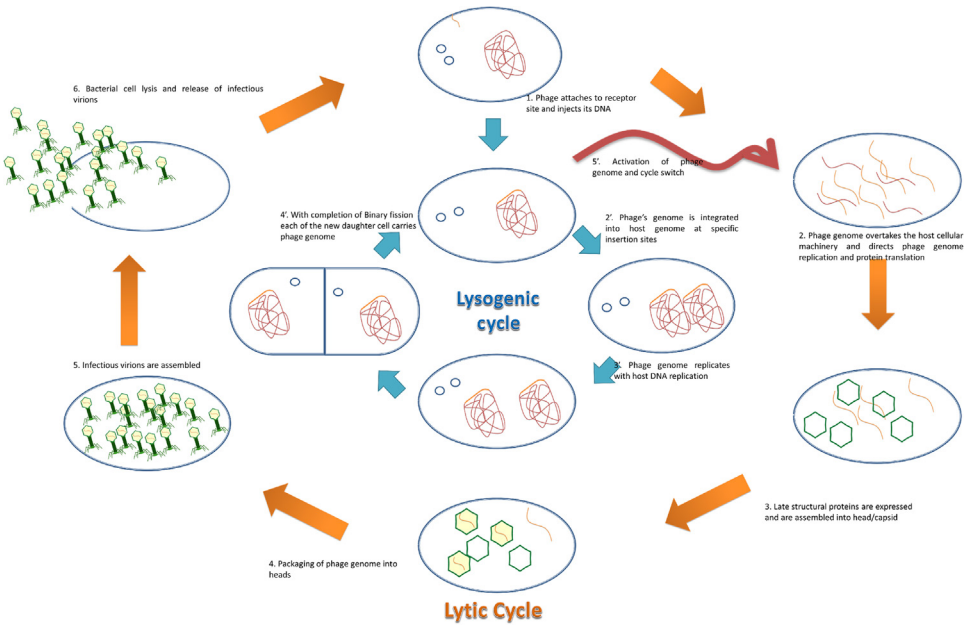

FIGURE 6.3 Lytic and lysogenic cycle of bacteriophage infection.

renders bacteria attenuated (Chiang & Mekalanos, 1999; Nesper, Schild et al., 2002) because of the role played by LPS in bacterial colonization and that is why LPS mutants fail to accumulate in the environment. In another study by Wei et al. (2010) it was revealed that resistant bacteria which evolve as a result of phage therapy are unable to play any significant role in the ecology or pathogenicity of *V. cholerae* as compared with the phage sensitive *V. cholera* (Wei et al., 2010). The phage resistance phenomenon, similar to antibiotic resistance, requires extra metabolic costs in terms of genes expression and energy consumption, etc., affecting the competitiveness of pathogens in the natural environment and suggesting they are avirulent at the site of infection (Wei et al., 2010).

Phage therapeutic experiments have been conducted against various clinically significant bacterial pathogens like *E. coli*, (Biswas, Adhya et al., 2002; Chibani-Chennoufi, Sidoti et al., 2004; Sabouri, Sepehrizadeh, Amirpour-Rostami, & Skurnik, 2017), *P. aeruginosa* (Waters, Neill et al., 2017), *Acinetobacter baumanii* (He, Hua et al., 2017), *Klebsiella pneumoniae* (Örmälä-Odegrip, Eriksson et al., 2015), *Enterococcus faecium* (vancomycin resistant strain, VRE), (Krisch, Prere, & Tetart, 2012; Mattila, Ruotsalainen, & Jalasvuori, 2015; Morris, Sulakvelidze, Alavidze, Pasternack, & Brown, 2011), *Streptococcus* spp. (Krisch et al., 2012; Seth, Geringer et al., 2013), and *Salmonella* spp. (Doss, Culbertson, Hahn, Camacho, & Barekzi, 2017; Endersen, O'Mahony et al., 2014).

6.7.3 Phage lysin enzymes

Bacteriophage lytic enzymes are the enzymes produced by the phages during their lytic life cycle (Ahiwale et al., 2012, Fenton, McAuliffe, O'Mahony, & Coffey, 2010). These enzymes are encoded by phage genomes (O'Flaherty, Ross, & Coffey, 2009, Schmitz, Schuch, & Fischetti, 2010). In the final step in the lytic cycle, progeny phages must be released from the infected host cell. This release is mediated by lysis or disruption of the host cell's membrane and cell wall. Lysin enzyme (peptidoglycan hydrolases) degrades the bacterial cell wall while holin creates pores in the cell membrane, both synergistically complete the last step of the lytic cycle (O'Flaherty et al., 2009; Wang, Smith, & Young, 2000).

Lysine enzymes are extensively reviewed by Fenton et al. (2010). Lysine enzymes are reported to have two domain structures consisting of an N-terminal catalytic domain and a C-terminal binding domain (Kretzer, Lehmann et al., 2007; Kutter & Sulakvelidze, 2004; Loessner, 2005; Loessner, Kramer, Ebel, & Scherer, 2002). The catalytic domain is classified into four groups de-pending upon cleavage sites: (1) N-cetylmuramidases (lysozymes); (2) N-acetyl-β-D-glucosaminidases (glycosidases); (3) N-acetylmuramoyl-L-alanine amidases; and (4) L-alanoyl-D-glutamate endopeptidases and interpeptide bridge-specific endopeptidases (Fenton et al., 2010). The C-terminal domain attaches the enzyme to its substrate by noncovalent interactions (Loessner, 2005).

Antimicrobial activity of phage-derived lytic enzymes against Gram-positive bacterial pathogens have been demonstrated in several studies (Cheng, Nelson, Zhu, & Fischetti, 2005; Fischetti, 2005; Loeffler, Nelson, & Fischetti, 2001; Nelson et al., 2001; Zimmer, Vukov, Scherer, & Loessner, 2002). Gram-positive bacteria, lacking an outer membrane, are vulnerable to bactericidal effects of these enzymes. These enzymes are reported to be more efficient, rapid, and lethal in comparison to antibiotics (Gilmer, Schmitz, Euler, & Fischetti, 2013). Unlike chemotherapeutic antimicrobials, lysins generally have target specificity, hence eliminating only the specific target pathogenic bacterial species while leaving normal commensal microflora (Daniel, Euler et al., 2010; Fenton et al., 2010; Fischetti, 2005).

6.7.4 Vaccines

Studies focusing on the development of protective vaccines against nosocomial infectious agents have been sought from as early as 1926 (Aoki, 1926, Christie, 1948; Fisher, Devlin, & Gnabasik, 1969). Those studies focused on the immunogenetic potential of a variety of antigens components of the type III secretion system (Sawa, Yahr et al., 1999), outer membrane proteins F (Mansouri, Gabelsberger et al., 1999), flagellar protein components (Christie, 1948), lipopolysaccharides antigens (Young, Meyer, & Armstrong, 1973), live attenuated mutant (Priebe et al., 2003), and killed whole-cell vaccines (Fisher et al., 1969). Stanislavsky & Lam (1997) and Knirel, Bystrova, Kocharova, Zaehringer, & Pier (2010) have discussed *P. aeruginosa* antigens in detail (Knirel, Bystrova et al., 2010; Stanislavsky & Lam, 1997). A heptavalent O antigen LPS vaccine (*Pseudogen*, by Parke Davis and Co.) was developed against *P. aeruginosa* and was found to be effectively protective against fatal infections in burns and cancer patients but exhibited more toxicity with poor efficacy in cystic fibrosis and leukemia patients (Haghbin, Armstrong, & Murphy, 1973; Luo, Lin, Gao, Zhang, & Zhang, 2014; Pennington, Reynolds, Wood, Robinson, & Levine, 1975). The development of recombinant live attenuated vaccines have been attempted against different pathogens with varying degrees of success (Han, Bai et al., 2015; Jackson, Phalen et al., 1999; Moral, del Castillo et al., 1998; Priebe et al., 2003; Stocker, 1988). The deletion of a single gene (*aroA*) caused a metabolic drag on the pathogen's metabolism due to the inability of aromatic amino acid biosynthesis capabilities. Yet recent studies have shown single-gene deletion vaccines have limitations in comparison to the double-deletion mutants (Han et al., 2015; Hu, You et al., 2015; Peighambari, Hunter, Shewen, & Gyles, 2002; van Lier, Sha et al., 2014). Knocking out two different virulence genes was comparatively more effective than single-deletion mutants, yet this approach is not so suitable for pathogens with a higher number of virulence factors and mechanisms, that is, *P. aeruginosa* (Winstanley & Fothergill, 2009). Secreted and surface-exposed proteins hold significant immunogenic potential (Stanislavsky & Lam, 1997) and have been targeted for vaccine development against many bacteria, that is, *Burkholderia pseudomallei* (Hara, Mohamed, & Nathan, 2009), *E. coli* (Puohiniemi, Karvonen et al., 1990), *Neisseria meningitides* (Boslego, Garcia et al., 1995), *V. cholera* (Sperandio, Giron, Silveira, & Kaper, 1995), and *Chlamydia trachomatis* (Hayes, Conlan, Everson, Ward, & Clarke, 1991). Montor et al. used self-assembling protein microarrays to study the immunogenic potential of outer membrane proteins of *P. aeruginosa*'s with the incitation of Th17 response and protection against respiratory infection (Montor, Huang et al., 2009; Wu, Huang et al., 2012). Selecting proper adjuvants is vital for enhancing the efficacy of any peptide vaccine as indicated by studies using LPS in combination with peptide vaccines (Gilleland, Parker, Matthews, & Berg, 1984). Type III secretory system components incited immune response in mice when used after purification elevated antibody titer (Holder, Neely, & Frank, 2001). Other studies have also shown the prospective protective results of bacterial fimbriae (Bakaletz, Leake, Billy, & Kaumaya, 1997; Chen & Schifferli, 2003; Huang, Liang et al., 2003; Piller, Clemente et al., 2005). The application of genomic and proteomic data also revolutionized the conventional vaccine development strategies and resulted in the advent of a reverse vaccinology approach (Del Tordello, Rappuoli, & Delany, 2017). This approach relies on physiochemical and structural properties of potential peptide antigens for peptide or subunit vaccine development with much ease and accuracy (Rashid, Naz, Ali, & Andleeb, 2017). Development of *menB*, a universal vaccine against serogroup B meningococci, was a paradigm-shifting event (Giuliani, Adu-Bobie et al., 2006). This strategy filters out the prospective proteins with desirable qualities for a vaccine candidate from pathogen's proteome. These qualities are: ease of exposure and accessibility for host immune system (subcellular localization; outer membrane/secreted), constitutive expression, involvement in virulence (infection establishment and progression), and non-homology to human proteins (for avoiding autoimmune responses). Proteins with such qualities are computationally assessed for their immunogenic potential and epitopes are predicted within the whole protein sequence. In order to achieve the best performing epitopes, the screening criterion is set so as to be able to effectively bind to the maximum number of major histocompatibility complexes (MHC) class I and class II alleles (Naz, Awan et al., 2015; Rashid et al., 2017). This approach has been used for multiple pathogens (Chiang, Sung et al., 2015; Delany, Rappuoli, & Seib, 2013; Gan, Zhao et al., 2010; Talukdar, Zutshi, Prashanth, Saikia, & Kumar, 2014).

6.7.5 Antimicrobial nanoparticles

Silver has been used as an antiinfective therapeutic agent for a long time (Rizzello & Pompa, 2014). The most commonly used forms of silver include silver sulfadiazine, silver acetate, and silver nitrate (Barah, 2013). Silver has been used as an antiinfective in burn wounds infections and also as a preservative in pharmaceutical products (Atiyeh, Costagliola, Hayek, & Dibo, 2007; Politano, Campbell, Rosenberger, & Sawyer, 2013). Silver nanoparticles are considered as potential candidates for antimicrobial therapeutics due to their optimal efficacy and broad-spectrum activity (Rizzello & Pompa, 2014). Various studies have been conducted to evaluate the mode of action of silver nanoparticles considering the variable effects of physiochemical characteristics, that is, shape, size, and surface of the nanoparticles. Silver nanoparticles of smaller diameter (1−10 nm) are reported to adhere to bacterial cell membranes with greater affinity compared with nanoparticles of bigger diameter, thus disturbing membrane activities (Morones, Elechiguerra et al., 2005). Various bacterial pathogens have been subjected to silver nanoparticles for bactericidal effects (Birla, Tiwari et al., 2009; Fayaz, Balaji et al., 2010; Kalishwaralal, BarathManiKanth, Pandian, Deepak, & Gurunathan, 2010; Kim, Kuk et al., 2007; Kim, San et al., 2010; Mohan, Oluwafemi et al., 2014; Shrivastava, Bera et al., 2007; Singh, Singh, Prasad, & Gambhir, 2008; Sondi & Salopek-Sondi, 2004). In another study a greater surface to mass ratio yielded higher antimicrobial efficacy. This bactericidal activity was reported to be associated with the generation and accumulation of reactive oxygen species in the microbial cells (Choi & Hu, 2008; Choi & Hu, 2009). In another study the effects of silver nanoparticles were evaluated on *E. coli* in both solid and liquid growth media. The results demonstrated higher activity in the solid medium at the same concentration, thus suggesting the bias of the experimental designs used in the studies (Sondi & Salopek-Sondi, 2004).

Despite the "miraculous" efficacy of silver nanoparticles, the role of the chemical environment can drastically reduce the bioactivity of silver. Interaction with chloride, bromide, and iodide anions may lead to the formation of either silver salts as precipitates/clusters or water-soluble ionic complexes. The formation of water-insoluble precipitates or clusters will reduce the access to the microbial cell, while on the other hand improved solubility will increase the bioavailability and efficacy. Thus depending upon the solubility of the main product, the effectiveness of silver may increase or decrease (Gupta, Maynes, & Silver, 1998). Another concerning issue is the resistance to heavy metals by the microbes. These resistance mechanisms have been exploited for environmental protection as bioremediation approaches (Barkay & Schaefer, 2001; Collard, Corbisier et al., 1994; Kamika & Momba, 2013; Malik, 2004; Nies, 1999; Sar, Kazy, Paul, & Sarkar, 2013; Silver & Phung, 1996; Silver, 1994). These resistance or tolerance mechanisms against silver and other metals may affect the antimicrobial activity of metal nanoparticles (Gupta, Phung, Taylor, & Silver, 2001, Percival, Bowler, & Russell, 2005, Silver & Phung, 2005; Silver, Phung, & Silver, 2006). In a study, the resistance against ionic silver was conferred by pyocyanin, a redox active virulence factor produced by *P. aeruginosa* (Muller & Merrett, 2014).

In another study by Randall et al. prolonged exposure to sliver did not yielded any resistance in *S. aureus* (Randall, Oyama, Bostock, Chopra, & O'neill, 2012). As discussed earlier in the case of antibiotic usage, the resistance mechanisms were already present in nature which received a massive opportunity to spread due to the extensive usage of antibiotics.

6.7.6 Thinking outside the box

The innate immune system is the vanguard acting as both the first line of defense as well as the rapid response force to neutralize immunological threats. Although nonspecific in its nature, this vanguard constitutes highly specialized components performing different duties throughout the human body which can be triggered within hours (Bruce Alberts, Lewis, Raff, Roberts, & Walter, 2002). Dual oxidase 1 (DUOX1) is one of the membrane proteins expressed in respiratory epithelial cells (Ameziane-El-Hassani, Morand et al., 2005). It utilizes $NADPH_2$ to synthesize hydrogen peroxide which is required by lactoperoxidase (LPO) for the generation of thiocyanate (Fischer, Lennemann et al., 2011; Rada & Leto, 2010). Hypothiocyanite is an antimicrobial oxidant produced in pulmonary surface liquid at the apical surface of the epithelial lining (Wesley, Bove, Hristova, McCarthy, & van der Vliet, 2007). Apart from functional association with LPO, DUOX1 has been reported to play a potential role in tracheal wound repair (Wesley et al., 2007) and be involved in excessive mucus generation (Shao & Nadel, 2005). Induction of DUOX1 is observed to be achieved by inflammatory cytokines (Harper, Xu et al., 2005; Rada, Lekstrom, Damian, Dupuy, & Leto, 2008) and bacterial stimuli (Gattas, Forteza et al., 2009). During *P. aeruginosa* pulmonary infections, after sensing the presence of the pathogen via Toll-like receptor (TLR)−mediated signaling, epithelial expression levels of DUOX1 are increased in order to achieve higher levels of hypothiocyanite (Rada & Leto, 2010; Raoust, Balloy et al., 2009). With the secretion of pyocyanin, a redox

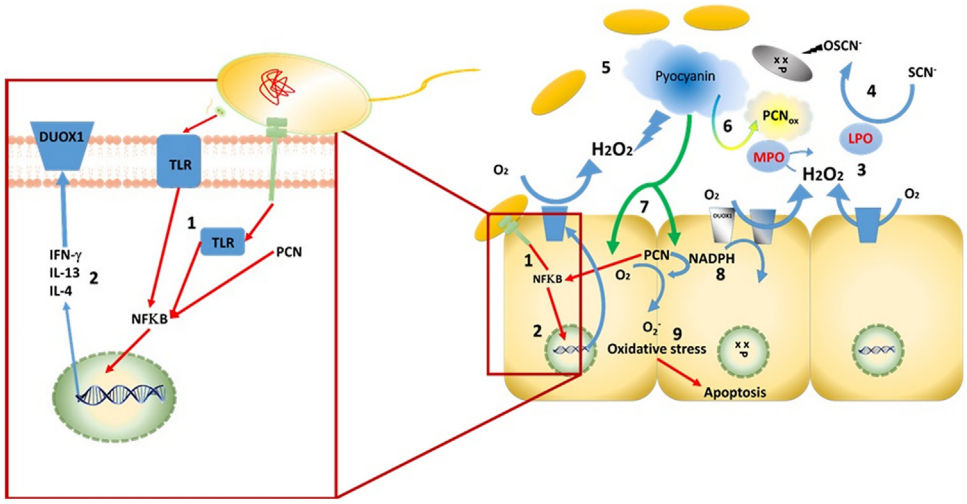

FIGURE 6.4 Host – PA Redox Duel ; (1) Activation of TLR via PA LPS, flagella, Type 3 Secretion System components. (2) Expression of inflammatory cytokines and activation of Duox1. (3) Production of H2O2in extracellular environment by Duox1 using NADPH. (4) Generation of Hypothiosynate for killing the infecting pathogen. (5) Secretion of Pyocyanin by *Pseudomonas aeruginosa*. (6) Peroxidase enzymes (LPO, MPO) oxidize PCN to detoxify in the presence of H2O2. (7) Diffusion of PCN across plasma membrane into the cell. (8) Generation of ROS by PCN using NADPH, thus competing with Duox1 and consequently inactivation of Duox1. PCN also increases the cellular levels of NFKB thus affecting gene expressions for immune-modulatory action. (9) Cell damage and potential induction of death.

active virulence factor, by *P. aeruginosa* the infection turns into a redox duel. Both the PCN and LPO DUOX1 systems are capable of neutralizing each other. PCN before gaining entry into the host epithelial cells is vulnerable to oxidation by peroxidase enzymes which diminishes its cytotoxicity. This enzymatic inactivation requires a plentiful supply of H_2O_2 which comes from DUOX1 (Reszka, Xiong et al., 2012). Once inside the cytoplasm, PCN readily consumes $NADPH_2$, thus reducing the substrate for DUOX1 (Rada et al., 2008). The outcome of this redox warfare between the host and the pathogen determines the course of infection (Fig. 6.4).

Studies have reported the reduced expression of DUOX1 in cystic fibrotic patients, clearly indicating the protective role against persistent *P. aeruginosa* infections (Marvig, Sommer, Molin, & Johansen, 2015, Minarowski, Sands et al., 2008; Moskwa, Lorentzen et al., 2007). Adopting strategies which can boast such innate immune mechanisms can also help in the management of superbug infections.

6.8 Conclusion

In this review the broader picture regarding the current status of drug resistance in microbial pathogens has been presented. The discovery of penicillin marked the dawn of a new era of antimicrobial therapeutic agents. But since the very first clinical administration of antibiotics, bacterial pathogens began to evolve and to survive. The extensive selection pressure faced by bacteria has not only resulted in the emergence but also the spread of resistance between normal susceptible microflora. The identification and development of novel drug targets and therapeutic agents is required to focus on cellular targets with the least selective pressure and to disarm the pathogens of their virulence factors. The different approaches reviewed above are at different levels of development and treatment. But the most important concern is the careful administration of the currently available and future antibacterial therapeutic agents.

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Further Reading

(2017) Global *priority list of antibiotic research bacteria to guide research, discovery, and development of new antibiotics*. World Health Organization.

Chapter 7

The benefits of active substances in amphibians and reptiles and the jeopardy of losing those species forever

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7.1 Introduction

In the long course of biological evolution, biodiversity has been increasing and diversifying via numerous biotic interactions, from predator and competitive to mutualistic and symbiotic (Futuyma, 2010). Through a specific evolutionary "arms race," interactions between either predator−prey or herbivore−plant species have resulted in the emergence of unique weaponry, that is, active biological substances synthesized in specialized cells of species involved in these interactions. Biologically active substances make their host-producers nonedible, passively venomous, cryptotoxic (such organisms synthesize active compounds in specific tissues and organs to repel predators; they are usually vividly colored), or actively venomous (i.e., phanerotoxic species which have specific organs such as fangs, stings, etc. for the active input of venom from glands into the predator's or prey's body) (see in Crnobrnja Isailović, Milojković, & Macura, 2015). Clearly, the preciousness of the world's biodiversity is reflected in the numerous natural products of wild species. During their cultural evolution, humans have been observing and examining biologically active substances synthesized by wild species occurring in their surroundings, which resulted in the development of "natural medicine," "alternative medicine," or "biomedicine." Medicinal compounds synthesized by many wild species offer solutions for an increasing number of health problems which Anthropocene humans have faced.

Many microorganisms have developed resistance to antibiotics. In the last two decades their number has been increasing (de Azevedo Calderon & Stábeli, 2011; Li et al., 2006). Some alternative treatment techniques, such as manufactured peptides, are somewhat expensive and "economically unfeasible" (de Azevedo Calderon & Stábeli, 2011; Marr, Gooderham, & Hancock, 2006). However, a number of life forms are sources of bioactive molecules that could be used as antibiotics (de Azevedo Calderon & Stábeli, 2011) for patients that receive immunosuppressive drugs and are therefore more prone to bacterial and fungal infections, especially when some antimycotics are toxic for humans (Clarke, 1997). This mostly includes patients undergoing major surgery, or suffering from diabetics, AIDS, and cystic fibrosis (Clarke, 1997).

7.2 Amphibians

Amphibians were the first vertebrates to invade land. They have relatively thin and permeable skin, a morphologically, biochemically, and physiologically complex organ that primarily protects them from unfavorable factors of the environment, but also has other functions necessary for their survival (Clarke, 1997; de Azevedo Calderon & Stábeli, 2011). Amphibian skin contains cutaneous glands that have many important roles in respiration, water regulation, temperature control, reproduction and offspring care, antipredator, antimicrobial, and antifungal defense (Clarke, 1997; de Azevedo Calderon & Stábeli, 2011; Toledo & Jared, 1995). Due to its role in respiration, amphibian skin must be moist, which makes it susceptible to bacteria and fungi; it is suspected that glands and their secretion evolved as a defense against these microorganisms (Habermehl & Preusser, 1969; Habermehl, 1995). Besides mucous glands providing a moist

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coating, amphibian skin also contains granular glands that are the site of synthesis of a wide range of chemicals to provide protection against bacterial and fungal infections as well as against predators (Clarke, 1997; Gomes et al., 2007). These glands may be scattered over the skin surface or arranged in clusters, forming compact glands such as the enlarged neck or parotid glands found in many tailed amphibians (e.g., salamanders) and anurans (Clarke, 1997; Gomes et al., 2007).

7.2.1 Secretions of the anuran skin

Compounds produced by anurans in their granular glands are quite numerous and diverse in chemical composition, but most of them include biogenic amines (adrenaline, noradrenaline, bradykinin, or histamine), steroids (bufadienolides and bufotoxins), alkaloids (batrachotoxin or tetrodotoxin, mostly in the Dendrobatidae family), peptides and proteins (such as bombesin and bombinins, found in *Bombina* species; Erspamer, 1988; Simmaco, Kreil, & Barra, 2009), and some uncharacterized/undefined toxins from the genus *Atelopus* (Gao et al., 2010; Kowalski, Marciniak, Rosiński, & Rychlik, 2018). The skin of the Japanese toad *Bufo japonicus* contains some other steroids—cardenolides—that affect the heart rate (Rodríguez, Rollins-Smith, Ibáñez, Durant-Archibold, & Gutiérrez, 2017). Bufogenines and bufotoxins also affect the heart by increasing the strength of the heart beat and decreasing the heart rate (Clarke, 1997).

Chen and Kovaříková (1967) reported that so-called "bufagin" toads have been used in ethnomedicine all over the world. For example, toads were considered healing agents (Habermehl, 1995). In China, there is a tradition of using Ch'an Su (Senso in Japan), a secretion from the parotid glands of native toad species (*Bufo gargarizans*, *Bufo melanostictus*), as a medicine for local inflammations, sinusitis, toothache, and canker sores (Chen & Kovaříková, 1967; Wang et al., 1991; Yoshida, Kamano, & Sakai, 1976). Bufadienolides are the components of Ch'an Su that have the greatest biological activity (Cunha Filho et al., 2005). A major component of bufadienolides is bufalin (Gao et al., 2010; Gella et al., 1995; Gomes et al., 2007). According to Rodríguez et al. (2017), this compound was isolated for the first time from the secretion of *Bufo marinus* (now *Rhinella marinus*) in 1912. In 2013 two other active compounds, bufotalin and bufotoxin, were extracted from *Bufo bufo* secretion. Other substances like cinobufagin and cinobufotoxin were listed later in Ch'an Su and this folk medicine was the source of further research. In addition, other components included are marinobufagin (Gao et al., 2010; Shimada, Ishii, & Nambara, 1986), hellebrigenin and telocinobufagin (Gao et al., 2010; Gella et al., 1995; Shimada et al., 1986), arenobufagin (Gao et al., 2010; Gella et al., 1995; Shimada et al., 1986; Tashmukhamedov et al., 1995), bufotalinin (Shimada et al., 1986), gamabufotalin (Gao et al., 2010; Tashmukhamedov et al., 1995), 19-hydroxybufalin, and bufalin-3-*O*-sulfate (Gao et al., 2010).

Today, Ch'an Su is applied in oriental medicine in clinical therapy for heart stimulation, in the treatment of tuberculous fistula, diuresis, neurodermatitis, toothache, as an antitumor compound, and as a painkiller (Wang et al., 1991; Yang et al., 2015). Some of the properties of toad secretions recognized so far are: cardiotropic, neurotropic, antibiotic, antitumor, radioprotective and immunomodeling (Tashmukhamedov et al., 1995). Some components, like arenobufagin, seem to have potent antitumor activity (Zhang et al., 2013). Cinobufagin also shows great anticancer effects on multiple types of lung cancer cells, demonstrating a higher selectivity between normal and cancer cells when compared to bufalin and arenobufagin (Peng et al., 2017). A similar effect is seen in Huachansu (cinobufacini), a sterilized extract from *B. gargarizans* skin mixed with hot water, also widely explored (Wang et al., 2010). It is used as an injection for detoxification, painkiller, a bone marrow proliferation promoter, immunity enhancer, antiviral agent, etc. (Yang et al., 2015). Yuan et al. (2016) reported that cinobufacini is an effective agent against cancer, with low toxicity and few side effects.

7.2.2 Amphibian species around us: hidden producers of valuable compounds

There are many species of amphibians that can be recognized as producers of valuable biologically active compounds. To enable a quick and informative insight into the preciousness of these "live biochemical laboratories" the following sections will summarize the available facts on their biology and the pharmacological value of secretions produced by a few common European anuran species.

7.2.2.1 Bombinatoridae

These are small toads, whose heads and bodies have a dark-colored dorsal side, while the ventral side of their throats and their bellies is vividly, aposematically colored. When stressed, these toads expose their bellies in a specific posture (behavior known as "unken reflex") (Arnold & Ovenden, 2002).

Many peptides and small proteins have been isolated and characterized from the secretion of *Bombina* toads, displaying different pharmacological, antimicrobial, or inhibitory activities (Gomes et al., 2007; König, Bininda-Emonds,

& Shaw, 2015; Simmaco et al., 2009). These include bombinins, bombesin, thyrotropin-releasing hormone, BSTI, and Bv8 (Simmaco et al., 2009). Bombinins (bombinin-like peptides) and bombinins H (H for hydrophobic and hemolytic) are the two families of antimicrobial peptides produced in the skin of *Bombina* toads that have not been detected in other amphibian genera (Simmaco et al., 2009). The best constituent of *Bombina* skin secretions known so far is bombesin (Bn), which was originally extracted from *Bombina bombina* and *Bauhinia variegata* (Anastasi, Erspamer, & Bucci, 1971). It was later observed that bombesin closely resembles two mammalian Bn-related peptides, gastrin-releasing peptide and neuromedin B (Erspamer, 1988), and many studies have been published describing the various pharmacological activities of bombesin and its homologues (Erspamer & Melchiorri, 1973; Erspamer, 1988; Gonzalez, Moody, Igarashi, Ito, & Jensen, 2008). Bombesin displays a stimulant action on a number of vascular and extravascular smooth muscles, such as intestinal, uterine, and urinary tract smooth muscle, as well as on gastric acid secretion in several species of mammals (Erspamer, Erspamer, Inselvini, & Negri, 1972). It also has an effect on the kidneys, with activation of the renin−angiotensin system (the hormone system that regulates blood pressure and fluid balance) and stimulates the erythropoietin release (Erspamer & Melchiorri, 1973). It was observed that bombesin mimics a mammalian gut−brain neurotransmitter, causing the suppression of feeding in experimental animals (King, 1991).

Bombesin has been shown to have a stimulating effect on smooth muscle derived from various parts of the human gastrointestinal tract (Corazziari et al., 1982). In vivo studies in humans have shown the inhibitory effect of bombesin on the mechanical activity of the small intestine and a stimulating effect on gallbladder contraction. Intravenous infusion of bombesin in humans results in increased serum gastrin levels and increased lower esophageal sphincter pressure; it also affects calcium metabolism. Bombesin stimulates the release of gastrin, pancreatic polypeptide, glucagon, gastric inhibitory polypeptide, angiotensin, and prostaglandins; in contrast it causes a fall in parathyroid hormone levels and reduces plasma glucose concentrations (Corazziari et al., 1982; Ghatei et al., 1982). Another potential use of bombesin can be found in clinical application regarding human tumors, including those of breast and prostate. These tumors express increased levels of the gastrin-releasing peptide receptor (GRP-R), which means that this receptor is a potential target for the use of bombesin-like peptides as molecular carriers of cytotoxic drugs or diagnostic radionuclides, for diagnosis and therapy (Maina, Nock, & Mather, 2006).

The secretory glands of *Bombina* toads also produce several other peptides with different biological activities that can also be found in other organism groups (Simmaco et al., 2009). These peptides may be of interest because of their similarities to mammalian hormones and neurotransmitters; there have been several cases where the description of an amphibian peptide has played a key role in the isolation of its mammalian analogue (Rosengren, Daly, Scanlon, & Craik, 2001). The thyrotropin-releasing hormone (TRH), a peptide first isolated from a mammalian hypothalamus that encourages the secretion of pituitary thyroid-stimulating hormone in mammals and is widely distributed throughout the brain of vertebrates, is present in the skin of the *Blatta orientalis* (Yasuhara & Nakajima,1975).

7.2.2.2 *Fire-bellied toad (*Bombina bombina*)*

7.2.2.2.1 Distribution, biology, threats

This species inhabits lowland areas of central, eastern, and southeastern Europe, including the Balkan peninsula. The northern border of its distribution includes Denmark and southern Sweden. In the Balkans this species inhabits lowland parts of the bank of the River Sava and the Danube, as well as lowland parts along their tributaries, going as far south as eastern Greece and European Turkey (Sillero et al., 2014).

The largest specimens do not exceed 5 cm in total length (Fig. 7.1). They have rough, coarse skin with many small serous glands. Their belly is black and intersected with small reddish, orange or yellow−orange markings, and tiny white dots (Fig. 7.2).

It is mostly found in permanently stagnant waters and hibernates on land, beneath stones, and in logs. Fire-bellied toads can be found in aquatic habitats from spring to autumn, and in surroundings nearby. The female produces a relatively small amount of eggs per year, up to 446, deposited in a few separate clumps (Arnold & Ovenden, 2002; Rafinska, 1991). It is globally considered as a common and not threatened anuran species, but it is listed as one of the species of conservation concern in Europe (Agasyan et al., 2009a). It is exposed to negative anthropogenous habitat alterations resulting from urban development, industry, intensive agriculture, water pollution, biological resource use, and the spread of amphibian pathogens (Agasyan et al., 2009a).

7.2.2.2.2 Medical importance

The trypsin inhibitor BSTI found in the skin secretion of *B. bombina* is an inhibitor of trypsin and thrombin; it is also called *Bombina* skin trypsin/thrombin inhibitor (Mignogna et al., 1996; Rosengren et al., 2001). A small protein named

FIGURE 7.1 Fire-bellied toad, dorsal view. *Photo: Jelka Crnobrnja-Isailović.*

FIGURE 7.2 Fire-bellied toad, belly pattern. *Photo: Jelena Ćorović.*

Bv8 was found in the skin secretion of *B. bombina* and *B. variegata* (Mollay et al., 1999). Two of its mammalian homologues have been described. In mammals, members of this family bind to two G-protein-coupled receptors and have different biological effects (Simmaco et al., 2009). For example, they induce contraction of smooth muscle in the gastrointestinal tract, they can induce increased sensitivity to pain, and they can also change behavior, such as feeding, drinking, and circadian rhythm (König et al., 2015; Mollay et al., 1999; Negri, Lattanzi, Giannini, & Melchiorri, 2007). It is suggested that these peptides are involved in hematopoiesis and in inflammatory and immunomodulatory processes because of the high expression level of human Bv8/PK2 in bone marrow, lymphoid organs, and leukocytes (Negri et al., 2007).

*7.2.2.3 Yellow-bellied toad (*Bombina variegata*)*

7.2.2.3.1 Distribution, biology, threats

The yellow-bellied toad is distributed in hilly and mountainous regions of Europe, from central France in the west, through its central parts and north of the Apennine peninsula, to the Czech Republic, Poland, Ukraine, Hungary, and Romania in the east, and also throughout the Balkan peninsula (Sillero et al., 2014).

This toad is similar in size and appearance (albeit sometimes somewhat larger) to the fire-bellied toad (Fig. 7.3). Its belly is yellow, intersected with dark flanks, while the dorsal side is sometimes paler than in the previous species and without prominent black spots (Fig. 7.4).

FIGURE 7.3 Yellow-bellied toad, dorsal view. *Photo: Jelena Ćorović.*

FIGURE 7.4 Yellow-bellied toad, belly pattern. *Photo: Jelena Ćorović.*

It inhabits predominantly small, shallow, and often temporary stagnant aquatic habitats or still parts of mountainous streams and rivers. The female produces up to 170 eggs per year, distributed in several small clumps (Arnold & Ovenden, 2002). The yellow-bellied toad is also considered to be a globally common and not threatened species but is listed as one of the species of conservation concern in Europe (Kuzmin et al., 2009). It is exposed to negative anthropogenous habitat alterations resulting from urban development, industry, mining, quarrying, construction of diversion minihydropower plants, as well as of biological resource use, lack of traditional farming, and the spread of amphibian pathogens (Kuzmin et al., 2009).

7.2.2.3.2 Medical importance

Studies on the antimicrobial and hemolytic properties of its skin secretions date back more than 40 years, when a peptide called bombinin possessing these activities was identified (Csordas & Michl, 1970). After that a fraction was isolated containing several peptides related to bombinin. This peptide group was named bombinins and it showed antibacterial but no hemolytic properties (Simmaco et al., 1991). Additionally, another group of hydrophobic peptides possessing hemolytic activity, bombinin H, was also isolated from *B. variegata* (Mignogna, Simmaco, Kreil, & Barra, 1993). Bombinins were found to be active against Gram-positive (*Bacillus megaterium* Bm11, *Staphylococcus aureus* Cowan1) and Gram-negative (*Escherichia coli* D21, *E. coli* D22, *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa* ATCC 15692) bacteria as well as against *Candida albicans*, but with no hemolytic activity. Bombinins H generally have lower antibacterial but higher hemolytic activity (Mangoni et al., 2000; Mignogna et al., 1993; Simmaco et al., 2009). It is interesting to note that the presence of isomers of these peptides and their related functional differences and

mechanisms of activity may possibly prevent the development of bacterial resistance (Mangoni et al., 2000). Moreover, skin secretions of *B. variegata* contain various insulin-releasing peptides, including three forms of bombesin. This finding may be useful for future studies and the possible exploitation of antidiabetic agents from natural sources (Marenah et al., 2004).

7.2.2.4 Bufonidae

This is a family of short-legged, robustly built toads which can be found in many different habitats on all continents except Australia (although one species has been introduced there). Their reproductive modes vary from egg-laying in aquatic environments to ovoviviparous and viviparous reproductive systems (Pough et al., 2015). Bufonids produce strong skin toxins, which have the potential to cure some diseases.

7.2.2.4.1 European common toad (*Bufo bufo*)

Distribution, biology, threats The European common toad is one of the most widespread anuran species in Europe. The only places where it does not occur are Ireland, Corsica, Sardinia, Balearic Islands, Malta, Crete, and some small islands (Sillero et al., 2014). It is a robust and large toad (up to 15 cm snout-to-vent length) with rough skin and prominent parotid serous glands (Fig. 7.5). The dorsal side of the body is brown and has a variety of darker blotches, while the belly is pale, often with brown marbling (Arnold & Ovenden, 2002).

Primarily its habitat is forested areas, but it can also be found in many other habitat types, from 0 to 3000 m in altitude (Agasyan et al., 2009b). The female produces anything from a few thousand to more than 10,000 eggs per reproductive season, arranged in double strings (Tomašević, Cvetković, Aleksić, Miaud, & Crnobrnja-Isailović, 2008). Although it is one of the commonest amphibian species in Europe, the European common toad is listed as one of the species of conservation concern in Europe (Agasyan et al., 2009b). It is exposed to negative anthropogenous habitat alterations resulting from urban development, industry, mining, quarrying, construction of diversion minihydropower plants, but also from biological resource use, and the spread of amphibian pathogens and invasive species (Agasyan et al., 2009b). Recent studies indicate an apparent population decline in several European countries (Jovanović & Crnobrnja-Isailović, 2019; Petrovan & Schmidt, 2016).

Medical importance The common toad seems to be a potentially good species for application in human medicine. Secretion of the adult's skin had very strong antimicrobial activity on *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus faecium* (DSM 13590) bacteria, while some moderate activity was also observed against *Staphylococcus aureus* (ATCC 6538P) (Nalbantsoy, Karış, Yalcin, & Göçmen, 2016). Additionally, some cytotoxic activity was recorded against multiple strains of cancer cells, such as A549 human alveolar adenocarcinoma, Caco-2 human colon colorectal adenocarcinoma, HeLa human cervix adenocarcinoma, MDA-MB-231 human mammary gland adenocarcinoma, MPanc-96 human pancreas adenocarcinoma, PC-3 human prostate adenocarcinoma, and U-87 MG human glioblastoma astrocytoma (Nalbantsoy et al., 2016). Some recent research reported that *B. bufo* tadpoles have greater quantities of active compounds in their bodies than adult individuals (Bókony et al., 2016; Üveges et al., 2017). Also it was revealed that compound content varies from population to population over geographical regions and that tadpoles produce more compounds in larger amounts when

FIGURE 7.5 Common toad, an adult specimen. *Photo: Jelka Crnobrnja-Isailović.*

competitors are more abundant (Bókony et al., 2016). In ponds where chances of desiccation are lower, tadpoles produce more bufadienolides (Bókony et al., 2016). It is suggested that *B. bufo* tadpoles synthesize their compounds de novo (Üveges et al., 2017): the authors discovered that the quantity of bufadienolides increases until mid-aged larvae stage (developmental stage 34), then starts to decrease and becomes lowest after metamorphosis. Some components obtained from common toad tadpoles collected in the Pilis Mountains, Hungary were bufalin, arenobufagin, bufotalin, gamabufotalin, resibufogenin, and telocinobufagin (Üveges et al., 2017).

7.2.2.4.2 Green toad (*Bufotes viridis*)

Distribution, biology, threats This species inhabits mostly open habitats from southern Sweden, Denmark, and the Baltic states in the north of Europe, eastern France to the west, Germany, Italy, Austria, Slovenia, and Hungary in eastern/southeastern Europe, and in Malta (Sillero et al., 2014). This toad is smaller than the common toad (up to 10 cm snout-to-vent length), has a pale dorsal side of the body, covered with dark-green markings (Fig. 7.6).

Preferably, it inhabits lowlands and steppe-like habitats, but it can also be common in agricultural fields, gardens, and human settlements. The female produces up to 10,000 eggs per reproductive season, arranged in strings like those of the common toad (Arnold & Ovenden, 2002). Although widely distributed and officially not threatened, the green toad has been recognized as a declining species (Aghasyan et al., 2015). It is also listed as one of the species of conservation concern in Europe (Aghasyan et al., 2015). Evidenced threats include the negative impact of agriculture and aquaculture, pollution from industry, transport (roadkills), and forestry (Aghasyan et al., 2015).

Medical importance Cardiotonic and vasotonic effects are reported for secretions from the skin glands of *B. viridis* (Gomes et al., 2007). Also *B. viridis* skin secretions showed antimicrobial activity when diluted with distilled water, HCl, NH_4OH, or phosphate buffers of 4 and 7 pH (Dülger, Uğurtaş, & Sevinç, 2004). All extracts showed effects against *E. coli* ATTC 10536, *Klebsiella pneumoniae* UC57, *Listeria monocytogenes* ATCC 19117, *Salmonella typhi* ATCC 19430, *Mycobacterium smegmatis* CCM 2067, *S. aureus* ATCC 6538P bacteria cultures, and *Rhodotorula rubra* and *Saccharomyces cerevisiae* ATCC 9763 fungi cultures (Dülger et al., 2004). The most successful extract was the HCl one, while phosphate buffers had only a weak effect on *E. coli* (Dülger et al., 2004). The constitution of compounds obviously varies from population to population. Gella et al. (1995), conducting a study in the Khrakov region, reported that the main component of the *B. viridis* secretion was gamabufotalin, while Gao et al. (2010) reported arenobufagin and hellebrigenin, both in only moderate quantities. The concentration of compounds secreted by the green toad is not very high: gamabufotalin, bufalin-3-*O*-sulfate, 19-hydroxybufalin, bufotalinin, telocinobufagin, marinobufagin and bufain are its minor components (Gao et al., 2010). This species was therefore pronounced not to be suitable by Chinese Pharmacopeia for use in human medicine (Gao et al., 2010). Still, the antimicrobial effects of green toad secretion are something that might be useful in human medical treatments.

Skin secretions of *Bufotes variabilis*, until recently considered in *B. viridis* complex, showed very similar results against the same cancer cell lines (above) and bacterial strains *E. faecalis* (ATCC 29212), *E. faecium* (DSM 13590), and *S. epidermidis* (ATCC 12228) (Nalbantsoy et al., 2016). This gives hope that with additional research something

FIGURE 7.6 Male and female green toad in amplexus. *Photo: Jelena Ćorović.*

more useful might be salvaged from the secretion of local *B. viridis*. Adult green toads synthetize many useful anticancer compounds, but in small quantities. Focus might be shifted to tadpoles, if there is a chance that they are more productive than adults. Also, there could be variations in the quantity of produced target compounds within populations, therefore both these toad species might be considered valuable for humans and put under a more efficient protection regime.

7.3 Reptiles

Reptiles were the first vertebrates to become independent of the aquatic environment in terms of reproduction (Pough et al., 2015). Reptiles' skin is modified into scales and the surface is composed entirely of β-keratin, which is uniquely produced by reptiles, while the interscalar space is composed of α-keratin (Sawyer et al., 2000). Venom delivery systems have evolved at least twice within Squamata (lizards and snakes) and there is a huge variety in morphology and effectiveness. The venom delivery system consists of the venom gland, surrounding muscles, ducts which transport venom from the glands, and fangs. The fangs of viperid snakes are very agile, hollow, tubular, with closed canals and can be erected by the rotation of maxilla (Kardong & Lavin-Murcio, 1993). Elapid snakes have fixed, relatively small fangs (with some exceptions). Venomous colubrid snakes have posterior, enlarged, often grooved teeth, which function as fangs (Jackson, 2003).

7.3.1 Snake venoms

Snake venoms are very complex mixtures of biochemical compounds with different effects, ranging from neurotoxic, cytotoxic, cardiotoxic, to myotoxic and many others. The most important components of snake venom include proteases which degrade structural proteins into peptides and amino acids and cause hemostatic disorder; acetylcholinesterases which hydrolyze acetylcholine and therefore disturb the transmission of nerve signals; phospholipases which hydrolyze phospholipids in the cell membranes affecting hemostasis; hyaluronidases which degrade hyaluronic acid and therefore help in spreading the rest of the venom throughout tissues; and disintegrins which inhibit integrins and block platelet aggregation and induce cell apoptosis (more in Lu, Clemetson, & Clemetson, 2005). Snake venoms have a long history of medical (with antitumor, antimicrobial, anticoagulating, and analgesic effects) and cosmetic use. The most famous one is of course the ACE inhibitor (captopril) which was obtained/extracted/derived from the venom of *Bothrops jararaca* (an enzyme which converts angiotensin I to the vasoconstrictor substance, angiotensin II) and this medicine represented an important discovery in cardiovascular medicine (Hayashi & Camargo, 2005).

7.3.1.1 Viperidae

The family of vipers harbors only 9% of all snake species but they inhabit nearly all terrestrial ecosystems of the world except Australia and Antarctica (Pough et al., 2015). This family of venomous snakes has many advanced traits; vipers are also characterized by their advanced venom delivery mechanisms and they produce highly complex, potent, natural toxins which are an important contributor to the global public health problem of venomous snakebite (see Maritz et al., 2016). They are highly threatened by continuous degradation, fragmentation, and loss of suitable habitats, by overexploitation (harvest for venom supply), direct persecution, and invasive species (listed and reviewed in Maritz et al., 2016).

Viper venom mostly consists of proteolytic and thrombin-like enzymes, hyaluronidases, phospholipases, disintegrins, crotactin (in crotalid snakes), viperatoxin (in snakes of genus *Vipera*), inorganic ions, glycoproteins, and amines, which work synergistically to subdue prey and start digestion (Vyas, Brahmbhatt, Bhatt, & Parmar, 2013). Viper venom mainly causes tissue degradation and disturbance of hemostasis (Meier & Stocker, 1991). Many compounds of viper venom have been used in human medicine and they are consistently the subject of numerous medical studies. For example, the medicine called ancrod is a thrombin-like enzyme from *Calloselasma rhodostoma* venom, and it is used in the treatment of ischemic strokes, HATT syndrome, deep vein thrombosis, and peripheral occlusive diseases; TSV-PA is plasminogen activating enzyme from *Trimeresurus stejnegeri* venom, used in the treatment of myocardial infarction, pulmonary embolism, stroke, different thromboses, and cancer (review in Mukherjee, Saikia, & Thakur, 2013). Recently, viper venom has been the subject of many studies on its antitumoral activity. For example, two phospholipases A2 from *Cerastes cerastes* venom, CC-PLA2-1 and CC-PLA2-2, inhibit cancerous cell adhesion and migration, similarly to phospholipase A2 from *Macrovipera lebetina* venom (MVL- PLA2) (reviewed in Calderon et al., 2014).

7.3.1.1.1 Nose-horned viper (*Vipera ammodytes*)

Distribution, biology, threats This species is mostly confined to the Balkans and neighboring regions (see Crnobrnja-Isailović & Haxhiu, 1997; Sillero et al., 2014; but see also Agasyan, Avci et al., 2009). It is characterized by apparent genetic structuring (Čubrić, Stamenković, Ilić, & Crnobrnja-Isailović, in press; Ursenbacher et al., 2008). This is one of the largest European vipers with a defined triangular head and a distinctive horn on the top of its snout and a zig-zag pattern on the dorsal side of the body (Arnold & Ovenden, 2002) (Fig. 7.7).

The nose-horned viper inhabits dry, often rocky habitats, including open woodland and scrub, sand dunes, hillsides, screes, stone walls, traditionally cultivated land, gardens, and vineyards (Agasyan, Avci et al., 2009). Although common in its range, its global population status is declining (Agasyan, Avci et al., 2009). Main threats are exploitation for venom supply, the pet trade, and it is generally persecuted by humans (Agasyan, Avci et al., 2009; Jelić et al., 2013).

Medical importance According to Expert Committee (2016) *V. ammodytes* is listed as a venomous snake species of greatest medical importance in Europe.

The subspecies *V. a. ammodytes* (*Vaa*) is the most common viperid snake in the Balkans. In one single bite, *V. a. ammodytes* can release about 20 mg of poison, rich in proteins, of which neurotoxin, cytotoxin, and hemotoxin are the most important (Gopčević, 2001). It also has large amounts of enzymes that are proteolytic (metalloproteinases or serine proteinases) or hydrolytic (phospholipases, L-amino acid oxidases) (Sajević, Leonardi, & Križaj, 2013). *V. a. ammodytes* is 20 times more common than *V. berus*, and it is the most dangerous of the European vipers producing hemotoxic, myotoxic, neuro-, and cardiotoxic effects (Karubova et al., 2016; Latinović et al., 2016). Georgieva et al. (2008) recently reported that the *V. a. ammodytes* snake venom proteome comprises more than 100 different proteins that can be grouped into nine protein families, with both enzymatic and nonenzymatic activities. By proteomic analysis the

FIGURES 7.7 Nose-horned viper can have different color variants: (A) color variant 1 and (B) color variant 2. *Photo: (A) Tijana Čubrić and (B) Jelka Crnobrnja-Isailović.*

presence of the following protein classes was shown: monomeric and heterodimeric Group II phospholipase (PLA2s), snake venom serine proteinases (SVSPs), snake venom metalloproteinases Group I, II and III (SVMPs), L-amino acid oxidases (LAAOs), cysteine-rich secretory proteins (CRISPs), disintegrins (Dis), and vascular endothelial and neural growth factors (VEGFs/NGFs) (Georgieva et al., 2008). Among the most toxic components of *V. a. ammodytes* venom isolated so far are ammodytoxins (Atxs), which are presynaptically neurotoxic secretory phospholipases A2 (Brgles et al., 2014; Križaj, 2011).

Enzymatic proteins in* V. a. ammodytes *proteome. *LAAO* (L-amino acid oxidase, EC: 1.4.3.2) catalyzes the stereospecific oxidative deamination of an L-amino acid substrate to an α-keto acid, along with the production of ammonia and hydrogen peroxide via an amino acid intermediate. Liberated hydrogen peroxide may be involved in the many toxic effects of LAAO, including hemorrhage, hemolysis, edema, apoptosis, antibacterial activity, as well as the regulation of platelet aggregation. The enzyme is present in significantly high concentrations in venom and a variety of related biological activities have been reported for isolated sv-LAAOs, including cytotoxic, apoptotic, platelet aggregation effects, edema, and bactericidal and antiparasitic activities. The enzyme is postulated to be a toxin (Zuliani et al., 2009).

SVSPs (snake venom serine proteases) are extensively studied toxins mainly affecting the hemostatic system, by inhibiting components of coagulation cascade. SVSP are classified in the clan PA, subclan PA(S), family S1 (chymotrypsin), subfamily A of the proteolytic enzymes. According to substrate features, these are considered to be trypsin-like enzymes and cleave peptide bonds following Arg or Lys at P1 position; SVSPs catalytic mechanism includes a highly reactive serine residue (Ser 195, chymotrypsinogen numbering) and thus they could be inhibited by serine-modifying reagents such as phenylmethyl sulphonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). They show a wide spectrum of biological activity: activation of plasminogen, thrombin-like activity; some are venom serine proteinase-like proteins, or snake venom serine proteases, or they could activate Factor V (Solange & Serrano, 2013).

SVMPs (snake venom metalloproteases, EC: 3.4.24) are a subgroup of reprolysins and belong to the metzincin family. Their classification is based on their different domain structures, presented as follows: P-I (SVMPs with only a metalloprotease domain), P-IIa to P-IIe (containing metalloprotease and disintegrin domains; they might be present as dimers or as only dimeric disintegrins), PIIIa to PIIIc (containing metalloprotease, disintegrin-like and cysteine-rich domains; can also be present as dimers), and finally PIIId, formally known as PIV (containing the PIII structure and two C-type lectin domains connected by disulfide bonds) (Fox & Serrano, 2010). The composition of most viper venoms includes at least 30% SVMPs, suggesting their potentially significant roles in envenomation-related pathogenesis such as bleeding, intravascular clotting, edema, inflammation, and necrosis. SVMPs are the primary factors responsible for local and systemic hemorrhage (Gutiérrez, Escalante, Rucavado, & Herrera, 2016). They have a Zn^{2+} ion in the catalytic site; this ion's activity can be inhibited by chelation of the Zn^{2+} ion with chelating agents such as EDTA or 1,10-phenantroline.

Nonenzymatic proteins in* V. a. ammodytes *proteome. *CRISPs* (cysteine-rich secretory proteins) are single-chain glycoproteins with molecular masses ranging from 20−30 kDa. They display 16 highly conserved Cys residues that form eight disulfide bonds. They are found exclusively in vertebrates and have broad diversified functions. Recent studies reveal that CRISPs are widely distributed in snake venoms and that they inhibit smooth muscle contraction and cyclic nucleotide-gated ion channels (Boldrini-França et al., 2017; McCleary & Kini, 2013; Sunagar, Johnson, O'Brien, Vasconcelos, & Antunes, 2012). CRISPs are a promising class of snake venom components that can be employed for future cancer drugs, due to the negative regulation of angiogenesis (Yamazaki & Morita, 2004).

SNACLECs (common C-type lectins) are nonenzymatic proteins that bind carbohydrate in a calcium-dependent manner. In snake venom, lectins are classified into true C-type lectins (CTLs) and snaclecs (also known as C-type lectin-like proteins or CLPs); they lack the carbohydrate-binding loop present in true C-type lectins and consequently do not bind sugars (Clemetson et al., 2001; Morita, 2005).

PIs (protease inhibitors) are proteins or peptides capable of inhibiting the catalytic activity of proteolytic enzymes. Serine protease inhibitors (SPI) are the largest and most widely distributed superfamily of PIs, best characterized by their Kunitz-type inhibitors. Their wide distribution is probably due to their abundance in several organisms. Venom Kunitz-type proteins play important roles in envenomation by inhibiting serine proteases acting in living processes, such as the hemostatic system (Choo et al., 2012; Mourão & Schwartz, 2013) or by blocking potassium channels (Harvey & Anderson, 1991) or both (Stubbs & Bode, 1995; Yang et al., 2014). SPIs exert thrombin-like activity by stimulating fibrinolysis and activating platelet aggregation (Chen et al., 2001).

VEGFs (vascular endothelial growth factor) are proteins which regulate blood vessel formation and vascular permeability. VEGF snake venom toxin vammin was identified. Vammin is a homodimeric protein similar to other members of the VEGFs and possesses short C-terminal positively charged tails that bind heparin. Vammin induces the synthesis and secretion of perlecan via VEGF receptor-2 in cultured human brain microvascular endothelial cells. Some authors

suggest its application in proangiogenic therapies (Olsson, Dimberg, Crueger, & Claesson-Welsh, 2006; Suto, Yamazaki, Morita, & Mizuno, 2005; Yamazaki & Morita, 2006; Yamazaki, Takani, Atoda, & Morita, 2003).

7.4 Why is it important to maintain these wild species viable?

The contemporary biodiversity crisis strongly impacts amphibians and reptiles. According to the overview of the proportions of threatened species within each of the more comprehensively assessed groups (shown in Fig. 7.2) of the IUCN Red List summary statistics, 40% of amphibians species are in threatened IUCN categories, while reptiles are doing little better (34% of selected reptiles, for example, marine turtles, sea snakes, chameleons, crocodiles, and alligators) (https://www.iucnredlist.org/resources/summary-statistics). Adequate population studies are missing for many of those species. The negative effects of human changes of environment are summarized in four major anthropogenic threats: the first three (habitat degradation, fragmentation, and destruction; overexploitation of natural populations; and the introduction of allochtonous species into ecosystems) result in a decrease of population size and eventually extinction of many autochtonous species. This induces the fourth threat: the chain of species extinction. There are also negative effects of contemporary climate change and extinction of ecosystems' top predators, which additionally jeopardize biological diversity on all scales. Additionally, in many parts of the world humans have a negative attitude toward amphibians and reptiles which has been inducing continuous persecution, deliberate killing, and habitat destruction of species belonging to these two vertebrate groups (Anthony et al., 2006; Böhm et al., 2013).

In both groups, a number of species synthesize complex substances that have a role in antipredator defense and/or immobilization of prey. These active substances are the result of millions of years of biological evolution, where evolutionary factors have been fine-tuning biochemical effects, leading to the more efficient adaptation (or natural product in this case). There is no laboratory in the world that can get results of the same quality. For new adaptations to emerge, there are three necessary prerequisites: genetic potential, heterogeneous environment, and time. Environmental heterogeneity could be spatial, temporal, or combined, and includes both abiotic and biotic components of environment. Abiotic heterogeneity implies variation of many factors such as temperature, humidity, precipitation, salinity, composition of chemical elements in the habitat, etc. Biotic heterogeneity refers to the richness of species interactions: predation, competition, mutualism, commensalism, symbiosis, etc. Time means that new genetic variants must be transferred to the next generation, with these being dependent on the ability to survive and reproduce. To provide appropriate background for the maintenance of the existing and the emergence of some future outcomes of species evolutionary potential, humans must preserve Earth's biological diversity. Biological evolution is a continuous process where populations of wild species adapt to changing conditions via natural selection. The higher the diversity of surrounding living organisms and their interactions is, the richer the expression of genetic potential in the form of biochemical, physiological, morphological, and behavioral adaptations will be.

Throughout the world there are many wild species threatened with extinction, whose biological value is still unknown; among them are also amphibians and reptiles. The importance of conservation of their populations is emphasized here, not only because of the functional role these species play in ecosystems (Petrovan & Schmidt, 2016), but also because they represent unique natural laboratories that produce valuable remedies for various health issues. The medicinal effects of those biochemical compounds are yet to be discovered.

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Chapter 8

Human genetic diversity in health and disease

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8.1 Genetic diversity in humans

Human genetic diversity is a genetic difference that exists among individuals and between populations, including changes ranging from single nucleotide polymorphisms to large genome rearrangements and epigenetic changes as well. According to widely cited data, human genetic diversity is estimated to be around 0.1%, meaning that any two individuals differ on average at about 1 in 1000 nucleotides (Andolfatto, 2001), although some more recent results suggest a higher rate of genetic diversity in human population (Redon et al., 2006). This area is a subject of great interest as it can shed light on human history and evolution and is of vital importance in improving health prospects. Genetic differences between individuals and populations can affect their susceptibility to disease, resistance to infection and pathogens, and their response to pharmaceutical treatments. Understanding and tracing those differences is vital in not only understanding the basis and mechanisms of various diseases but also in discovering new drug targets and designing new drugs. The pattern of genetic variation within and between human populations is the result of demographic history and evolutionary processes that shaped the human genome as an adaptive response to pathogens, the environment, and dietary challenges for tens of thousands of years (Balaresque, Ballereau, & Jobling, 2007; Jorde, Watkins, & Bamshad, 2001). Building on the already existing body of knowledge, novel technologies and methodologies in genomics will enable us to understand these differences better and utilize the knowledge in biomedicine, from the discovery of new disease-causing genes to designing new therapies.

Infectious diseases are one of the strongest evolutionary pressures that have affected and sculpted the human genome over time. Infections by different pathogens have been and still continue to be the leading cause of death in many parts of the world, thereby acting as a force that strongly selects on genetic variants that confer disease resistance. This idea was first suggested by Haldane who noted that heterozygosity for certain hematological disorders is associated with resistance to malaria (Haldane, 1949). Malaria is caused by the mosquito-borne parasites from the genus *Plasmodium* that infects red blood cells and is responsible for the majority of deaths from infectious diseases worldwide (Greenwood & Mutabingwa, 2002; Snow, Guerra, Noor, Myint, & Hay, 2005). Malaria is one of the oldest diseases that affected mankind (Hartl, 2004) and given the length of its presence in the human population and its morbidity and mortality before reproductive age, it has acted as a strong selective force on the human genome and, in certain parts of the world, it still continues to do so (Fumagalli et al., 2011). Malaria is an evolutionary force that has led to favoring genetic variants responsible for conditions such as sickle cell anemia, thalassemia, and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Mockenhaupt et al., 2004; Pauling & Itano, 1949; Piel et al., 2010). Mutations underlying these conditions provide different levels of protection against malaria when present in a heterozygous state and have been consequently selected at higher frequencies in areas where malaria was or is still endemic, despite the fact that in a homozygous state they result in fatal blood disorders. A mutation in the gene for the β-chain of hemoglobin, resulting in hemoglobin S, provides almost 90% protection against the mortality of malaria, while in the homozygous state gives rise to sickle cell anemia (Taylor, Parobek, & Fairhurst, 2012). The thalassemias are blood disorders caused by mutations in either the α- or β-chain of hemoglobin, with around 50% reduction in the risk of malaria shown for both heterozygotes and homozygotes for some α thalassemias (Allen et al., 1997). Mutations in the G6PD gene which lead to the G6PD deficiency are linked to hemolytic anemia upon exposure to certain infections, foods, or compounds

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(Beutler, 1994; Beutler, 2008). This deficiency offers approximately 50% protection against malaria infection to carriers (Gilles et al., 1967) but it is interesting to note that at the same time it causes adverse reaction to the antimalarial drug primaquine (Alving, Carson, Flanagan, & Ickes, 1956).

Naturally occurring resistance to HIV infection serves as an example where discovery of genetic resistance can serve as a basis for potential therapy. This resistance is caused by a 32-base deletion in β-chemokine receptor 5—CCR5 (CCR5-Δ32), the cell surface receptor that serves as a coreceptor for HIV. The presence of this deletion prevents the expression of the receptor on CD4 + T cells and the entry of the virus, thus providing complete HIV immunity in homozygous carriers and limited protection against disease progression in heterozygous carriers (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996). Finding that the prevention of CCR5 expression renders cells insensitive to HIV infection has provided the grounds for therapies based on stem cell transplantation. This approach was first proven successful more than a decade ago, when a patient who received blood stem cells from a CCR5-Δ32 homozygous donor remained without any signs of HIV infection (Allers et al., 2011). A similar approach in another patient has been reported more recently (Gupta et al., 2019), highlighting the potential of developing HIV therapies based on the reduction of CCR5 expression and the medical relevance of identifying naturally resistant variants in populations.

Cystic fibrosis (CF) is the most common autosomal recessive disorder in the Caucasian population with a carrier frequency greater than 2% (Farrell, 2000). It is a complex multisystem disorder caused by mutations in the gene that encodes cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel present in epithelial cells of many organs (Berger et al., 1991; Hwang & Kirk, 2013; Sheppard & Welsh, 1999). Clinical manifestations of the disease include lung disease, pancreatic insufficiency, endocrine disorders, gastrointestinal and hepatobiliary problems (Milosevic et al., 2013; O'Sullivan & Freedman, 2009). To date, more than 2000 sequence alterations of the CFTR gene have been identified (Cystic fibrosis mutation database statistics), including missense, frameshift, splicing, and nonsense mutations; large and in-frame deletions or insertions; promoter mutations and presumed nonpathological variants, with most of them being population specific with low worldwide distribution (Tsui, 1992). Out of all these mutations only around 130 of them can be defined as clearly disease causing (Sosnay et al., 2013), while others can have varying or no consequences. Based on their functional consequences—expression, trafficking, stability, and function of the CFTR protein—mutations are classified into six groups (Haardt, Benharouga, Lechardeur, Kartner, & Lukacs, 1999; Ratjen et al., 2015; Welsh & Smith, 1993) with classes I−III usually associated with more severe forms of CF.

Mutations responsible for CF are closely associated with populations of European ancestry. The CF shows a defined geographical pattern, affecting 1 in 2500 individuals in populations of European descent (Castellani et al., 2010), around 1 in 15,000 individuals in the Middle East (Frossard et al., 1998), 1 in 40,000 in Indian populations (Powers, Potter, Wessel, & Lloyd-Still, 1996), and is very rare in African and East Asian populations (Yamashiro et al., 1997). The most frequent CFTR mutation is F508del, a 3 base-pair deletion of phenylalanine 508, which is estimated to be around 50,000 years old (Morral et al., 1994; Wiuf, 2001). The F508del mutation is present in around 85% of patients worldwide and has higher frequency in northern Europeans than in southern Europeans (De Boeck, Zolin, Cuppens, Olesen, & Viviani, 2014). Apart from F508del, none of the other CF mutations have a frequency greater than 5% and they all show great regional heterogeneity in frequency and distribution (Bobadilla, Macek, Fine, & Farrell, 2002; Ratjen et al., 2015). Due to its high heterogeneity and population distribution, CF also represents an example of disorder where ancestry data are important in establishing a proper diagnostic tool and screening for disease-causing mutations (Bobadilla et al., 2002; Nikolic et al., 2010).

One of the possible explanations as to why CF has persisted and was not eliminated during evolution is a heterozygote advantage against infectious diseases. The proposed candidate diseases include cholera, typhoid fever, and tuberculosis. The cholera and typhoid fever hypotheses are based on the mechanism of pathogenesis in these infections that would provide certain levels of protection for CF mutations carriers, however epidemiological data and geographical distribution of CFTR mutation do not show favor for this hypothesis (Crump, Luby, & Mintz, 2004; Högenauer et al., 2000; Rodman & Zamudio, 1991; van de Vosse et al., 2005). The tuberculosis hypothesis is, on the other hand, supported by both molecular and clinical data. Several reports have shown that incidence of tuberculosis is lower in patients with CF (Kilby et al., 1992; Smith, Efthimiou, Hodson, & Batten, 1984) and dysfunction of enzyme arylsulfatase in CF cells can protect against tuberculosis by depriving *Mycobacterium tuberculosis* of a nutrient necessary for the construction of its cell wall (Tobacman, 2003).

The development of agriculture and animal domestication around 10,000 years ago and the dietary changes that came with it have also had significant impacts in favoring certain traits in human populations (Dudd & Evershed, 1998). Lactose tolerance is one of the best-studied examples of nutritional adaptation (Hollox et al., 2001; Poulter et al., 2003; Tishkoff et al., 2007). Lactose is the main carbohydrate present in milk which, in order to be fully digested and

used as an energy source, needs to be hydrolyzed by a specific enzyme called lactase. In most mammals, including humans, activity of this enzyme is high only during infancy, the period when milk is the main source of nutrients, and declines before adulthood. This condition is known as lactose intolerance, an inability to digest lactose resulting in various gastrointestinal symptoms after milk consumption and is present in around two-thirds of the world's population (Ingram, Mulcare, Itan, Thomas, & Swallow, 2009; Storhaug, Fosse, & Fadnes, 2017). At first, it was believed that this intolerance is an exception from the norm (Auricchio, Rubino, Landolt, Semenza, & Prader, 1963). However, now we know that lactose persistence, retention of an active enzyme into adulthood and ability to digest lactose, is an acquired phenotype which resulted from adaptation to drinking milk of domesticated dairy animals. Frequency of lactose persistence varies across populations and has high prevalence in Europe, especially Northern Europe, and is present in certain parts of Africa and Asia, correlating with domestication of dairy animals and milk consumption. In Europeans, lactase persistence is the result of a single genetic variation (C/T-13910) in an intron of a lactase neighboring gene, MCM6 (Enattah et al., 2002), while in Africans it is a result of several different changes in the lactase gene (Ségurel & Bon, 2017; Tishkoff et al., 2007), thus serving as an example of convergent evolution where the same phenotype developed independently in response to environmental condition.

8.2 Epigenetic diversity in humans

Not all differences between individuals or populations can be explained by genetic differences and one of the mechanisms contributing to this diversity is epigenetics. The term epigenetics was first introduced by Waddington in the 1930s, describing the processes that are involved in embryology and genetics—"development as an epigenetic process" (Waddington, 1940). In the paper "The Epigenotype," Waddington explicitly defined epigenetics as an investigation regarding the relation between phenotypes and genotypes (Waddington, 1942). Since then, the meaning of the word has been modified and now we consider the term as depicting the processes that are involved in regulation of gene expression. The epigenetic regulation of gene expression guides developmental processes, but can also aid in the understanding of the differences between individuals that cannot be explained by genetic factors alone. The differences in the individual epigenomes can contribute to interpopulation differences of specific quantitative traits, but also contribute to disease development, progression, and response to therapy (Xiao, Cao, & Zhong, 2014). Epigenetic modifications are dynamic, reversible, and provide more rapid adaptive response to environmental changes, compared to genetic. There are several mechanisms that represent epigenetic control of gene expression: DNA methylation, microRNAs, and histone modifications.

DNA methylation is the first epigenetic mechanism to be discovered and to this day remains the best-studied one (Avery, Macleod, & McCarty, 1944). Methylation represents a process where the methyl group is covalently linked to cytosine in a specific CpG context at specific positions in the DNA, called CpG islands (Deaton & Bird, 2011). The process of DNA methylation is carried out by specific enzymes called DNA methyltranferases (DMNTs) (Chen & Riggs, 2011). The CpG islands are located in promoter regions of more than half of human genes (Vavouri & Lehner, 2012). Hypermethylated DNA regions are usually not transcriptionally active, while hypomethylation characterizes transcriptionally active regions. Different methylation patterns have been linked to developmental processes and cell fate decisions, but also to specific phenotypic traits, such as disease phenotype, development, progression, and response to therapy.

MicroRNAs (miRNAs) represent a class of small, 18−28-nucleotide-long, noncoding RNA molecules (Lee, Feinbaum, & Ambros, 1993; Macfarlane & Murphy, 2010). They play an important role in gene regulation, usually acting like repressors of gene expression (Wahid, Shehzad, Khan, & Kim, 2010). There is redundancy in function of miRNAs, where multiple miRNAs can influence the expression of a single gene, but also one miRNA can exert its action on several genes (Macfarlane & Murphy, 2010). They are important biomarkers for disease susceptibility and progression, but also represent an attractive target for potential therapy (Vidigal & Ventura, 2015).

Histone modifications represent covalent posttranscriptional modifications of histone proteins affecting gene expression by altering chromatin structure (Mariño-Ramírez, Kann, Shoemaker, & Landsman, 2005). Some of the posttranscriptional modifications of histones are methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation (Bannister & Kouzarides, 2011). The changes in histone modifications can lead to the open, transcriptionally active state of chromatin, or to the closed, repressed state where gene expression is locked. Enzymes that modify histones are designated as "writers" and "erasers." Writers add posttranscriptional modifications and erasers remove them (Gillette & Hill, 2015). There are not much data about the influence of histone modifications on human diversity. Some studies have shown that there is a connection between histone modification machinery and certain diseases such as hematological malignancies and solid tumors (Butler, Koutelou, Schibler, & Dent, 2012). There is a series of rare diseases that are

associated with mutations in histone modification enzymes, including Kabuki syndrome, X-linked mental retardation, and Kleefstra syndrome (Butler et al., 2012). These mutations can lead to alterations in gene expression and chromatin remodeling.

The most studied functions of epigenetic markers have been in the context of disease, but there are emerging data showing that methylation can affect phenotypic traits, outside the disease content. Differences between human populations in methylation patterns have been identified, leading to population-specific phenotypic traits. The study of Heyn and coworkers has shown a clear link between CpG sites differentially methylated between Caucasian-American, African-American, and Han Chinese-American individuals (Heyn et al., 2013). Using 439 different methylation sites the authors have been able to separate the distinct populations with respect to their geographical origins and to associate them with distinct phenotypic characteristics, such as appearance, drug metabolism, responses to external stimuli, sensory perception, and disease susceptibility. Importantly, local selective pressure was shown to induce the manifestation of epigenetic variants, exemplified by immune and xenobiotic response factors and their potential positive selection by differences in local pathogen and environmental pressure (Heyn et al., 2013). Some of the epigenetic differences identified were associated with underlying genetic changes, but some were not, suggesting that they could be used as epigenetic markers associated with natural variation in humans. Natural epigenetic diversity has been suggested as a key mechanism in microevolutionary processes due to its capability to create phenotypic variability within individuals and populations. It constitutes an important reservoir of variation potentially useful for rapid adaptation in response to environmental stimuli. The analysis of population epigenetic structure represents a possible tool to study human adaptation and to identify external factors that are able to naturally shape human DNA methylation variability. Publicly available epigenome-wide data were used in several studies to explore population-specific DNA methylation changes that occur at macrogeographic scales. Results from these analyses suggest that nutrients, UVA exposure, and pathogen load might represent the main environmental factors able to shape DNA methylation profiles. DNA methylation diversity is a source of variability in human groups at macro- and microgeographical scales and population demographic and adaptive histories, as well as the individual ancestry, actually influence DNA methylation profiles.

As mentioned above, epigenetic mechanisms drive development and contribute to human diversity, but also can be involved in all aspects of disease mechanisms, from susceptibility to response to therapy.

Epigenetics can influence several aspects of infectious diseases, such as resistance to infection, infection progression, and a host's response to infection. There have been noted differences in methylation of promoters between different ethnic groups (Caucasian-American, African-American and Asian-American) that are important for the penetrance of HIV infection (*HIVEP3*, *HTATIP2*, *CDK11B*), as well as enteropathogenic *Escherichia coli* and measles virus infection (FYN), and hepatitis B virus infection (HLA-DPA1) (Heyn et al., 2013). The hepatitis B infection risk, which is directly related to underlying genotypes and epigenetic marks, and the consequent enrichment of these in affected populations, is an illustrative example of the tight interplay between these two layers of organization (Heyn et al., 2013). In malaria, a complex infectious disease, the role of epigenetics is understudied, as it is in most other infectious diseases. Generally speaking, epigenetic regulation of specific host genes which provide the resistance to a pathogen or mediate immune response can influence the response to infection. Deficiency of pyruvate kinase enzyme is one of the most important factors that contributes to resistance to malaria and its epigenetic regulation may play an important role since it can lead to downregulation of the expression and disease resistance. Promoter methylation of the ABCB1 gene together with two polymorphisms can influence malaria susceptibility, prognosis, and response to therapy (Gupta et al., 2017). Inhibitors of chromatin-modifying enzymes that target the *Plasmodium* parasite have been considered as antimalarial drugs. Detecting immune system−related genes enriched at potentially epigenetically inherited CpG sites suggests that the impact of pathogens on their host's DNA profiles (Paschos & Allday, 2010) subsequently leaves footprints in the epigenome of their progeny (Heyn et al., 2013). These changes can lead to differences in infection susceptibility.

DNA methylation has been shown to have an important role in modulation of monogenic diseases with cystic fibrosis being one example. The methylation patterns in nasal epithelium of CF patients have altered methylation compared to non-CF nasal epithelial cells (Magalhães et al., 2018). These changes are found in regions of DNA where genes responsible for epithelial integrity and inflammatory and immune responses are located, and which are important for disease mechanism. Not only is the methylation pattern between normal and CF cells different, but there are differences in methylation between patients with different disease phenotypes, estimated by pulmonary severity. For example, the genes *HMOX1* and *GSTM3* in nasal epithelial samples and *HMOX1* and *EDNRA* in blood samples were differentially methylated between patient groups with different pulmonary severity. Also, lower DNA methylation levels at *GSTM3* were associated with the *GSTM3*B* allele, a polymorphic three base-pair deletion that has a protective effect in cystic fibrosis (Kho et al., 2018; Magalhães et al., 2017). Several miRNAs have been identified to influence CFTR protein expression, both wild-type and carrying F508del mutation. Increased expression of miR-145, miR-223 and miR-494

in vivo has been shown to correlate with decreased CFTR expression in bronchial epithelium of individuals bearing the F508del-CFTR mutation (Gillen, Gosalia, Leir, & Harris, 2011). Experimental modulation of miRNA expression confirmed the hypothesis supporting the view that deregulation of miRNA may affect CFTR biogenesis in CF cells (Noel & Leal, 2015). One of the characteristics of the airway of the CF patients is that their airway epithelial cells overexpress interleukin 8 (CXCL8). The mechanism leading to this overexpression is linked to epigenetic changes in these cells. The trimethylation of histone H3 at lysine 4 (H3K4ne3) and hypomethylation at CpG6 in CXCL8 promoter in CF cells compared to non-CF cell, leads to increased bromodomain 3 (Brd3) and Brd4 recruitment and enhancement of Nf-κβ and c/EBPβ binding to the promoter. Additionally, it has been shown that this could be used as a potential therapy target, because inhibitors of bromodomain reduced expression of CXCL8 in CF cells (Poghosyan, Patel, Clifford, & Knox, 2016).

There is a limited number of studies that have investigated the specific, functional role of miRNAs in lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). One study showed that miRNA-199-5p is the most upregulated miRNA in asymptomatic patients with α-1 antitrypsin deficiency, carriers of ZZ mutated alleles (Torres-Durán et al., 2018). Not only do these alterations cause the differences in gene expression that lead to disease phenotype modification, they also can lead to different responses to therapy (Magalhães et al., 2017). Asthma is a complex, heritable disease and genetic, epigenetic, and environmental factors all contribute to its development. The role of epigenetics in asthma development has been investigated in human populations, and also in several animal models. Alterations in epigenetic markers have been associated with environmental exposures relevant for asthma development, such as air pollution and smoking. Additionally, asthma phenotypes have been linked to several epigenetic changes. It has been shown that there is a connection between circulating miRNAs and lung function in asthmatic patients. Methylation has been shown to be an important factor in asthma development. The differences in 9 CpGs and 35 regions have been identified in newborns in relation to asthma development and could be further used as potential biomarkers of later asthma risk. In children, even more CpGs (179) and 36 regions have been discovered to be differentially methylated. The differences have been proved to be related to alterations in gene expression in most of these cases. Many of the genes with altered methylation are involved in immune responses that are crucial for asthma. Additionally, two identified genes, *IL5RA* and *KCNH2*, have been selected as targets for approved or experimental drugs. Childhood asthma has been shown to be linked to 59 differentially methylated regions in another study, and the genes annotated to the top 10 identified DMRs were *HOXA5*, *PAOX*, *LINC00602*, *ABCA7*, *PER3*, *CLCA1*, *VENTX*, *NUDT12*, *PTPRN2*, and *TCL1A* (den Dekker et al., 2019). The results from these studies and meta-analyses have confirmed that several differences in methylation patterns, although maybe subtle, together confer the higher risk of asthma development throughout the life course. The remodeling in asthma involves airway smooth muscle cells and this is a mechanism that is poorly understood, but has a potential to play a role in asthma therapy. The role of miR-142-3p has been suggested in regulating the balance between proliferation and differentiation of airway smooth muscle (ASM) cells in asthma (Bartel et al., 2018). Additionally, this miRNA has been linked to lung function changes (specifically forced expiratory volume, FEV1%). It targets genes that can be related to changes in FEV1%—*CCTN2* and *LRRC32*. Serum miRNAs are associated with lung function in asthmatics and could prove to be a biomarker for disease severity. The correlation between exacerbation clinical score has been shown for asthmatics with miR-146b, miR-206, and miR-720 expression (Kho et al., 2018). These three microRNAs were involved in NF-kβ and GSK3/AKT pathways. Histone modifications influence asthma risk and severity, but also response to therapy. Many histone modifications have been linked to regulation of inflammatory and immune responses, such as antigen-presenting dendritic cells (DCs), and cell-lineage commitment of T cells, which are key players in asthma (Kidd, Thompson, Barrett, & Baltic, 2016). H3K4me3 and H3K27me3 sites characterize gene expression of monocyte-derived DCs under inflammatory conditions as they transition from immature to mature antigen-presenting cells. Early onset of asthma has been linked to specific histone modifications H3K27me3/H3Ac/H3K9me3, in the region where the gene *ORMDL3* is located (Verlaan et al., 2009). Histone modifications influence therapy response in asthmatics, especially corticosteroids, which are one of the most commonly used drugs to control asthma. Patients with severe asthma have diminished corticosteroid sensitivity of peripheral blood mononuclear cells (PBMCs) when compared with patients with nonsevere asthma, associated with a reduction in HDAC activity that parallels the impaired corticosteroid sensitivity (Hew et al., 2006). It has been shown that environmental factors can influence epigenetics. One of the main factors is smoking. Additionally, many studies have shown that epigenetic, methylation changes can be transferred through generations (transgenerational epigenetic inheritance) after some environmental changes and/or challenges (Lane et al., 2003; Seisenberger et al., 2012). In the case of the previously mentioned asthma, a large cohort study has shown that there is an increased risk of development of asthma in granddaughters when grandmothers were smokers. This has been linked to differences in gene expression and some methylation patterns. Passive smoking impairs HDAC2 function via PI3K signaling activation, which could

contribute to corticosteroid-insensitive inflammation in children with severe asthma. This novel mechanism could be a treatment target in children with severe asthma and stresses the need for a smoke-free environment for asthmatic children (Kobayashi et al., 2014).

Epigenetics have been extensively studied in many cancers and have been linked to almost every aspect of this disease. Epigenetic mechanism are involved in cancer development, progression, and response to therapy. Additionally, several epigenetic marks have been found that can be used as early biomarkers for several types of cancers. Changes in DNA methylation are seen as a promising target for development of diagnostic, prognostic tools, as well as for development of novel therapies. Hypermethylation in cancers usually occurs in promoters of tumor suppressor genes leading to their inactivation, and hypomethylation is characteristic for regions of DNA where oncogenes are located (Pfeifer, 2018).

There is strong evidence that altered expression of small noncoding RNAs may influence the progression of solid tumors. The upregulated miRNAs in tumor cells are commonly considered to be oncogenic miRNAs (oncomiRs), which can silence the tumor suppressor genes. The miR-21 is a very widely studied oncomiR and has been reported at high expression levels in glioblastoma, pancreatic cancer, breast cancer, and colon cancer. The miR-183 (miR-183/182/96 cluster) has been linked to poor survival and overall prognosis of breast, colorectal, hepatocellular, and oral cancers, including tongue cancer (Supic et al., 2018). Meta-analysis of available data showed that one of the epigenetic characteristics of NSCLC is that miR-21 and miR-155 are upregulated, while miR-148a, miR-148b, and miR-let-7 are consistently downregulated and are associated with poor overall survival (Lamichhane et al., 2018). These miRNAs show potential as useful prognostic biomarkers in the diagnosis, treatment, and follow-up of NSCLC. There is also prognostic value in assessing global levels of histone modifications. Lower levels of H3K4me2 are associated with poorer outcomes in prostate, lung, and kidney cancer (Seligson et al., 2009). Lower levels of H3K18ac and H3K9me3 predict a poorer outcome in lung, breast, gastric, and prostate cancer (Elsheikh et al., 2009; Park et al., 2008; Seligson et al., 2005, 2009), whereas lower survival is seen for patients with lung cancer tumors expressing higher levels of H3K9ac (Song, Kim, Kim, Park, & Jang, 2012). Specific patterns of H3K9me are associated with clinical outcomes in acute myeloid leukemia (Müller-Tidow et al., 2010). Specific histone modifications are also present in NSCLC, such as an increased acetylation status for histone H2A, with an increased H3 global trimethylation. Likewise, in solid lung tumors from patients with NSCLC, an excessive acetylation level on both histone marks H4K5 and H4K8, as well as hypoacetylation on H4K12/H4K16, accompanied with H4K20me3, in both lung malignant precursor lesions and particularly SCC lesions, has been identified (Langevin, Kratzke, & Kelsey, 2015). Recent reports demonstrate that the combination of acetylated histone marks H2AK5ac/H3K9ac and H3K4me2 has a significant prognostic value in NSCLC (Brzeziańska, Dutkowska, & Antczak, 2013).

8.3 Translational potential of human genetic and epigenetic diversity

In the modern setting, understanding human genetic diversity has great translational value in biomedical research and the healthcare system as it is vital for setting the proper diagnostic and screening tools, as well as for establishing optimal treatment options. The concept and knowledge of human genetic diversity has helped in forming an approach in medicine termed personalized or precision medicine which goes beyond the "one size fits all" viewpoint. Personalized medicine aims to provide the optimal diagnostic and therapeutic strategies and to maximize drug efficacy and minimize adverse drug reactions for a specific individual based on the specific information about that individual. This includes lifestyle and environment as well as genetic background—pharmacogenetic and pharmacogenomic information. Pharmacogenetics and pharmacogenomics are two disciplines both studying the role of genetic factors in the action of particular drugs, their effectiveness and adverse reactions (Kalow, 2006; Weinshilboum, 2003). While pharmacogenetics is a term used for the role of single genes in studying responses to drugs, term pharmacogenomics, which came with recent developments in DNA sequencing techniques, is more comprehensive and takes into account the role of multiple genes or the entire genome. Advances in the field of transcriptomics, proteomics, and metabolomics have also had a significant impact on personalized medicine and their integration is vital in improving the future of this field.

Although the field of personalized medicine has been revolutionized with developments in genetics and especially in the postgenome era, the concept of differences between individuals in terms of susceptibility to illness or reactions to different agents has been recognized throughout history, acknowledging the role of the lifestyle as well as overall individual differences for which the cause was still unknown at the time (Sykiotis, Kalliolias, & Papavassiliou, 2006; Novelli, 2010; Dance, 2016). In ancient Greece, Hippocrates wrote about the individuality of disease and medicine not being absolute; he noted that physicians should choose the appropriate treatment depending on the patients' individual characteristics, such as different health status and lifestyle (Konstantinidou, Karaglani, Panagopoulou, Fiska, &

Chatzaki, 2017). According to the legend, it was Pythagoras who in the 6th century BCE noticed the connection between hemolytic anemia and fava beans ingestion, a condition now know to be caused by G6PD deficiency (Alving et al., 1956). At the beginning of the 20th century the discovery of blood groups is the event now believed to have marked the beginning of the study of human genetic diversity (Goldman & Schmalsteig, 2016; The Nobel lectures in immunology. The Nobel Prize for Physiology or Medicine, 1930, awarded to Karl Landsteiner. 'In recognition of his discovery of human blood groups', 1990). This period is also characterized with experimental developments in biochemistry and discoveries of several enzyme deficiencies, which were also important for setting the stage and forming the mindset about the inherent uniqueness of individuals. In 1902, based on his studying of alkaptonuria, Archibald Garrod proposed the idea of "chemical individuality" and later the concept of "inborn errors of metabolism" (1908) suggesting that defects in metabolic pathways could be behind the three other disorders as well—pentosuria, cystinuria and albinism (Burgio, 1986; Garrod, 1996; Knox, 1958). He was the first to connect Mendel's laws of inheritance with human disorders and the first to propose recessive inheritance in humans, with his work now being recognized as the foundation of medical genetics (Rosenberg, 2008). In 1931 it was noted that the ability to taste phenylthiocarbamide was genetically determined and due to a single recessive gene (Fox, 1932; Snyder, 1931). Arno Motulsky, who is now considered as a founder of pharmacogenetics, was the first to link inheritance to the response to drug therapy in 1957 (Motulsky, 1957). He discussed his idea based on the examples of sensitivity to the antimalarial drug primaquine linked to G6PD deficiency and adverse effects to the muscle relaxant suxamethonium chloride in anesthesia caused by pseudocholinesterase deficiency. While studying G6PD, Motulsky also noted that malaria was the selective force behind the frequency and geographic distribution of G6PD deficiency (Motulsky, Vandepitte, & Fraser, 1966; Siniscalco, Bernini, Latte, & Motulsky, 1961). The term pharmacogenetics was coined around 1959 by Friedrich Vogel and was established as a novel discipline by Werner Kalow's monograph in 1962 (Werner, 1962). This textbook defined pharmacogenetics as "pharmacological responses and their modification by hereditary influences." Since then, genetics has evolved greatly and genetic bases to many diseases and traits have been discovered, with continuous improvements in technology enabling us to further expand this knowledge. Clinical utility of pharmacogenetics and pharmacogenomics has been validated through several examples of successful targeted therapies including therapy for cancer and cystic fibrosis.

Cystic fibrosis represents one of the best examples where genetic information plays an important role in the choice of therapy and where personalized medicine has brought significant clinical improvement. Until recently, conventional therapy for CF was mostly focused on treating the symptoms and complications of the disease and improving lung function and nutrition. Currently, the focus in treatment strategy has been on drugs targeting the basic defects underlying the dysfunctionality of CFTR protein and aimed at correcting the defects. In the last decade small molecules correcting the CFTR function were introduced into clinical practice as treatment options that work on correcting the function of CFTR protein with a specific underlying mutation or mutation class. The first of these to be approved for clinical use was ivacaftor, which was identified from a cell-based high-throughput screening of more than 200,000 molecules (Van Goor et al., 2009) and which soon after showed improved lung function, weight gain, and quality of life in clinical trials (Accurso et al., 2010; Edgeworth et al., 2017; Quittner et al., 2015; Ramsey et al., 2011). Ivacaftor acts as a potentiator as it improves chloride secretion by increasing the opening time of CFTR channel at the cell surface and was at first indicated for use in patients with G551D mutation, a class III mutation that affects the gating of the CFTR chloride channel. Given that worldwide frequency of G551D mutation does not exceed 4% of all patients with CF, there was still a need for the drug that would target a larger number of patients. However, the successful application of ivacaftor in patients with G551D was a valuable example showing the significance of a targeted treatment approach, even if it benefits only a small subgroup of patients. Further clinical trials have led to expanding the application of the drug to treat people with one of 38 ivacaftor-responsive mutations in the CFTR gene thereby increasing the number of patients that could benefit from it (Rowe et al., 2017; Vertex; 2017 Press release, FDA approves KALYDECO® (ivacaftor) for more than 600 people ages 2 and older with cystic fibrosis who have certain residual function mutations, 2017).

Ivacaftor's success changed the field of CF therapeutics and prompted the search for drugs that would target more frequent mutations and benefit more patients. The similar high-throughput approach was used to develop a drug that would work as a CFTR corrector and increase the delivery of CFTR to the cell surface. Mutations leading to this defect are classified as group II mutations and include F508del, the most frequent cystic fibrosis−causing mutation, estimated to be present on more than 70% of chromosomes in patients with cystic fibrosis (Worldwide survey of the delta F508 mutation--report from the cystic fibrosis genetic analysis consortium, 1990). This approach led to identification of lumacaftor, which compared to ivacaftor showed less significant effect on improving CFTR function but still achieved the levels of correction necessary to reduce disease severity (Van Goor et al., 2011). This drug is currently approved in combination with ivacaftor and is indicated for patients homozygous for F508del mutation (Wainwright, Elborn, & Ramsey, 2015). However, defects underlying class II mutation are much more challenging to correct and improve and

although this drug combination improved multiple clinical end points in treated patients, the effect is much lower than the effect ivacaftor has in G551D patients, addressing the need for further development of CFTR modulators.

Personalized medicine in cancer treatment involves tailoring the therapy based on physiological, molecular, genetic, and epigenetic profiling of the specific tumor. Substantial progress has been made in defining genetic and epigenetic mechanisms of carcinogenesis which has helped in pinpointing the crucial events in developments of specific tumors. Characterizing these genetic alterations provides a basis for predicting the course of the disease and establishing tailored therapy that targets specific pathways active in a given tumor (Baehner et al., 2011).

Nonsmall cell lung cancer (NSCLC), which accounts for 85% of lung cancers (Sher, Dy, & Adjei, 2008), is one of the examples for personalized therapy in cancer which showed a lot of success. Treatments for lung cancer have progressed from traditional chemotherapy to more personalized approaches based on the molecular signature of the cancer, with important therapeutic targets in lung cancer being the activated epidermal growth factor receptor (EGFR) and EML4-anaplastic lymphoma kinase (ALK). Mutations in EGFR gene are present in approximately 10%−15% of NSCLC patients of European and Asian descent (Lynch et al., 2004; Paez et al., 2004), leading to EGFR activation and subsequent EGFR-driven tumor formation. Activated EGFR can be targeted by EGFR-tyrosine kinase inhibitors—gefitinib, erlotinib, and afatinib—whose effectiveness in NSCLC treatment has been shown in several clinical studies (Maemondo et al., 2010; Rosell et al., 2012; Wu et al., 2014). However, these drugs act only in a subset of patients harboring specific activating EGFR mutations, mostly exon 19 deletions and L858R point mutation in exon 21. Additionally, as a response to the treatment, tumors can develop a resistance mechanism, which is in majority of the cases caused by a T790M mutation within exon 20 of the EGFR gene (Thomas, Rajan, & Giaccone, 2012), which subsequently can be targeted by osimertinib, a third-generation EGFR TKI. Another powerful oncogene in lung cancer is EML4-ALK fusion gene which can also be therapeutically targeted. The ALK is a tyrosine kinase receptor whose constitutive activation happens primarily through the formation of fusion genes, with EML4-ALK translocation being the most common ALK gene rearrangement (Scagliotti, Stahel, Rosell, Thatcher, & Soria, 2012; Soda et al., 2007). Treatment options for ALK-positive lung cancer include tyrosine kinase inhibitors such as crizotinib, ceritinib, and alectinib (Larkins et al., 2016; Yoshida et al., 2016).

Cancer immunotherapy is an emerging cancer treatment whose major goal is to activate the immune system components to specifically kill tumor cells. A powerful approach in cancer immunotherapy is a chimeric antigen receptors (CAR)−T cell therapy which can be viewed as the ultimate personalized treatment as it uses the patient's own T cells to attack the tumor. In this approach the patient's T cells are engineered to express highly active T cell receptors that target tumor antigen. The CAR-T cell therapy revolutionized treatment of cancer and has shown significant promise in treatment of B cell lymphoma (Levin & Shah, 2019; Nair & Neelapu, 2018) with ongoing clinical trials evaluating its use in solid tumors.

The epigenetic-based therapies for lung cancer are mainly focused on enzymes that methylate DNA (DNMTs) and histone-modifying enzymes (HDAC), with several drugs currently in various phases of clinical trials. Some of them have been previously approved for the treatment of other malignancies. Although single epigenetic-based treatments show promise in in vitro studies, in clinical trials the single compound treatments have not yet given satisfactory therapy results, mainly because of the low efficiency and considerable side effects. Consequently, therapies involving epigenetic mechanisms are now focused on combinations with conventional chemotherapy, immunotherapy, or kinase inhibitors. Treatment of lung cancer patients with a combination of HDACi (entinostat) and DNMTi (azacitidine) has shown some success in clinical trials. Entinostat is a benzamidine HDAC inhibitor targeting class I HDACs 1 and 3, which are preferentially found in the nucleus and regulate histone acetylation. Azacitidine is a cytidine analogue which inhibits DNA methyltransferase upon incorporation into DNA. It is approved as a drug for myelodysplastic syndrome. This combination treatment has been most successful in patients with specific methylation signatures on four genes (*CDKN2a*, *CDH13*, *APC*, and *RASSF1a*). These genes are specifically silenced by hypermethylation in tumors and strongly associate with disease recurrence and death, both singly and in combination (Brock et al., 2008). In the mentioned clinical trial, after the combination treatment with azactidine and entinostat, hypomethylation of at least two of these genes was detected in patients who had better overall survival (Juergens et al., 2011). Reversion of cancer-specific epigenetic abnormalities by epigenetic-based therapy is a promising direction in cancer treatment as it enables reprogramming of cancer-characteristic traits which are potentially reversible.

We have presented an overview of several mechanisms and the best known examples of human diversity, as well as translational potential of the knowledge on human genetic diversity. New technologies have provided us with the means to investigate human diversity on a large scale, and already much data are available. Nevertheless, the interpretation of vast amounts of gathered information still poses a problem. The question of human diversity in health and in disease is very complex and many factors have to be taken into account when analyzing the available data. The obtained data

could help to reconstruct human demographic history and migration events more accurately, to understand why some genetic diseases have evolved and were maintained, as well as to better understand how genetic and epigenetic diversity shape phenotypic traits. Characterization of human genetic variation also holds great promise in various medical contexts, from disease susceptibility, mechanism, progression, to response to therapy and design of novel drugs.

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Chapter 9

Potential for cancer treatment: natural products from the Balkans

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9.1 Introduction

The Balkan Peninsula is very complex part of Europe, known as a crossroads of nations with different religious and cultural heritage. The diverse geographical configuration of this region has given rise to diverse and unique ecosystems. Numerous species from the Balkan Peninsula have been a vital part of folk medicine for centuries, and the traditional knowledge about the medicinal value of natural resources was shared among different populations and transferred through generations. Nowadays, our duty is to collect and scientifically exploit the knowledge gained by our ancestors for the survival and better health of future generations.

The aim of this chapter is to emphasize the natural sources from the Balkans that are vital for the isolation and identification of new compounds that could be developed as anticancer agents. In addition, we wish to draw the attention of the scientific community to species that should be preserved for the benefit of humankind. Although some of the described species have a wide distribution in the Balkans and rest of the world, others are native, endemic, and endangered due to their irresponsible exploitation. The species with the highest medicinal value are particularly overutilized. Therefore significant efforts should be undertaken to preserve those species and find means to use their medicinal potential in a sustainable manner (Neergheen-Bhujun et al., 2017).

Cancer is considered to be a complex disease that is harder to treat than diagnose or characterize by using molecular biology tools. Carcinogenesis is associated with many initial events which significantly affect normal cells such as exposure to the oxidative stress, inflammation, loss of immune surveillance, and the metabolic switch from oxidative phosphorylation to glycolysis (Griffiths, Gao, & Pararasa, 2017). However, cancer should be as curable as any other disease and enormous hope for hindering cancer progression was and still is devoted to traditional medicine and natural sources. It is well-known that the majority of anticancer drugs on the market were developed from chemical structures found in nature (Newman & Cragg, 2016).

Some of the species found in the Balkans were traditionally used to treat cancer: plants of the genus *Alnus*, the latex of *Euphorbia dendroides*, as well as aerial parts of the plants from the genus *Achillea*. Some of the plants whose ingredients showed significant anticancer potential were also traditionally used to heal other diseases: their antiinflammatory potential (plants of the genera *Alnus*, *Achillea*, and *Micromeria*), antioxidant characteristics (plants of the genera *Micromeria* and *Helichrysum*), as well as immunosupportive properties (plants of the genus *Sideritis*) were recognized centuries ago.

It is important to highlight that some of the compounds whose anticancer potential is presented in this chapter originate from endemic (*Sideritis scardica* Griseb., *Laserpitium ochridanum* Micevski, *Laserpitium zernyi* Hayek, *Micromeria dalmatica* Benth., *Micromeria fruticosa* Druce, and *Helichrysum zivojinii* Černjavski & Soška) and endangered (*Nepeta rtanjensis* Diklić & Milojević) species.

N. rtanjensis Diklić & Milojević is an endemorelict found only on Rtanj Mountain in southeastern Serbia, specifically localized on open limestone terrains in the oak forest. According to the categorization of the International Union for Conservation of Nature (IUCN), it is a critically endangered species (Diklić, 1999). The remaining number of growing plants is estimated to be 500−700 (Mijović, Popović, Mišić, & Karadžić, 2007). According to research conducted

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by the Institute of Biological Research "Siniša Stanković" in Belgrade, there is hope for cultivation and reintroduction of *N. rtanjensis* Diklić and Milojević into its natural habitats from where it will undoubtedly disappear in the near future (Nestorović et al., 2010).

9.2 Genus *Alnus*

The genus *Alnus* (alder) is a member of the Betulaceae family and includes monecious trees and bushes distributed throughout the Northern Hemisphere and Central America. Numerous pharmacological studies have reported antiinflammatory, antiobesity, and antioxidative effects of various *Alnus* species (Chung et al., 2006; Dahija, Cakar, Vidic, Maksimovic, & Paric, 2014; Kuroyanagi et al., 2005; Stevic et al., 2010). This genus is an abundant source of diarylheptanoids, biologically active secondary metabolites increasingly acknowledged for their potential medicinal value. These phenolic compounds have been shown to possess antibacterial (Lee, Lee, Kim, & Ahn, 2009), antiviral (Tung, Kwon et al., 2010), antioxidative (Matsuda et al., 1998; Tung, Kim et al., 2010), anticancer (Choi et al., 2008; Mshvildadze, Legault, Lavoie, Gauthier, & Pichette, 2007), and chemoprotective properties (Novakovic et al., 2013).

9.2.1 *Alnus incana* (L.) Moench

A. incana, also known as gray alder (Fig. 9.1), is a small deciduous tree or shrub found across the northern hemisphere, and native to large areas of northern and central Europe. In the Balkan region, it is mainly distributed throughout Serbia and Albania (Fig. 9.1) (Jovanovic, 1970). Oregonin (1,7-bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-*O*-β-D-xylopyranoside) is an open-chain diarylheptanoid glycoside with 3-carbonyl and 5-xylosyloxy groups, that it is abundantly found in the hydrophilic extracts from the bark of gray alder. This diarylheptanoid was shown to possess

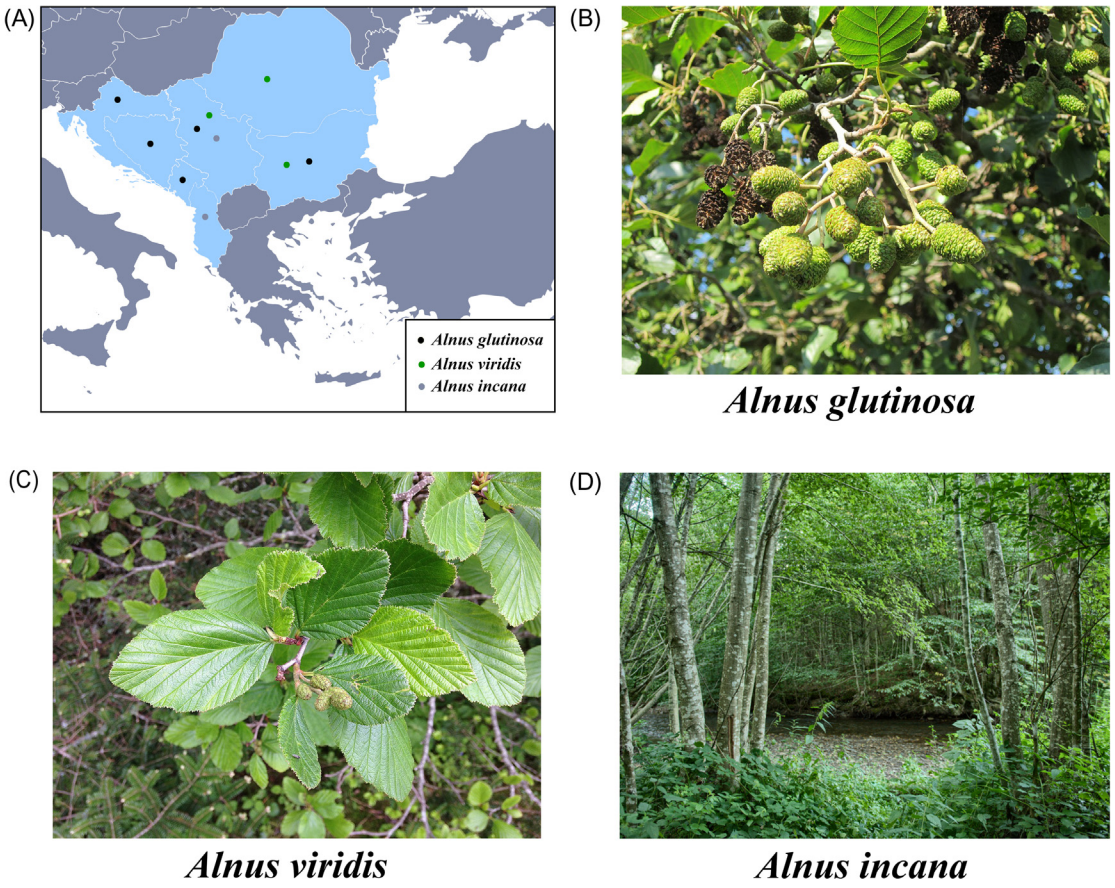


FIGURE 9.1 (A) Distribution area of *Alnus incana*, *Alnus glutinosa*, and *Alnus viridis* in the Balkans. (B) *Alnus incana* (gray alder). (C) *Alnus glutinosa* (black alder). (D) *Alnus viridis* (green alder).

antioxidative and antiinflammatory properties, and helps prevent obesity and associated metabolic disorders (Choi et al., 2008; Dinic et al., 2015; Lee, Lee, Chen, Ho, & Lin, 2005; Martineau et al., 2010; Tung, Kim et al., 2010). In a study which investigated the effects of oregonin in vitro on blood samples obtained from volunteers with metabolic syndromes (Telysheva et al., 2011), the total and low-density cholesterol and triacylglycerols levels were reduced after incubation with oregonin-containing ethyl acetate extract from *A. incana* bark. Another study found that oregonin altered DNA methyltransferases expression and mtDNA copy numbers in mouse embryonic fibroblasts in a manner which was cell genotype- and concentration-dependent (Krasilnikova et al., 2018). Molecular modeling indicated that oregonin fits the catalytic site of DNMT1, suggesting it could act as an inhibitor of DNMT1 enzymatic activity. These findings substantiate the potential of oregonin to regulate the epigenetic changes and genes involved in these processes.

9.2.2 *Alnus glutinosa* (L.) Gaertn

A. glutinosa (Fig. 9.1), also known as European alder or black alder, is a tree growing in the Mediterranean region and Europe, as well as western Siberia, the Caucasus Mountains, and southeastern Asia (Jovanovic, 1970). In the Balkans, it is found in Serbia, Croatia, Bosnia and Herzegovina, Montenegro, and Bulgaria (Fig. 9.1) (Jovanovic, 1970). Diarylheptanoids from the *A. glutinosa* bark collected in Serbia displayed chemoprotective activity in human nonsmall cell lung cancer cells, human keratinocytes, and peripheral blood mononuclear cells and antagonized the activity of the chemotherapeutics cisplatin and doxorubicin (Dinic et al., 2014). Treatment of cells with these compounds diminished mitochondrial fragmentation after doxorubicin application and cisplatin-induced free radicals by increasing Mn-SOD and HIF-1a mRNA expression (Dinic et al., 2014). Additionally, these diarylheptanoids protected human keratinocytes against doxorubicin-induced DNA damage and prevented doxorubicin-induced cell death via autophagy induction (Dinic et al., 2015). Diarylheptanoids platyphylloside, alnuside B, and hirsutenone, also isolated from *A. glutinosa* found in Serbia, showed considerable anticancer potential and were selective toward the lung cancer cells with the multidrug-resistant (MDR) phenotype (Novakovic et al., 2014).

Another group of diarylheptanoids was isolated from the black alder bark, including oregonin, platyphylloside, rubranoside A, rubranoside B, hirsutanonol, hirsutenone, hirsutanonol-5-*O*-β-D-glucopyranoside, platyphyllonol-5-*O*-β-D-xylopyranoside, aceroside VII, alnuside A, and alnuside B (Novakovic et al., 2013). All of the compounds, except oregonin and hirsutanonol, were found in Serbian species for the first time indicating significant chemodiversity of the black alder. The majority of the isolated compounds showed protective properties regarding chromosome aberrations in peripheral human lymphocytes in vitro and decreased DNA damage.

9.2.3 *Alnus viridis* (Chaix) DC

A. viridis (green alder) is a 3−5 m tall bush growing in the Alps and the Balkan Peninsula mountains, mainly in Serbia, Bulgaria, and Romania (Fig. 9.1) (Ball et al., 1964; Jovanovic, 1970). In Serbia, *A. viridis* ssp. *viridis* is distributed in the eastern regions, specifically on Stara Planina Mountain at 1300−2100 m (Jovanovic, 1970). A study compared structurally analogous diarylheptanoids from the green and black alder barks collected in Serbia and revealed that *A. viridis* diarylheptanoids induced a prominent apoptosis in human nonsmall cell lung carcinoma cells compared to their *A. glutinosa* analogues (Dinic et al., 2016). Compounds from the green alder prompted accumulation of intracellular superoxide anion and evidently reduced mitochondrial transmembrane potential. Although *A. viridis* diarylheptanoids exhibited greater cytotoxicity, their black alder analogues were more selective toward cancer cells compared to normal keratinocytes, which would make them more favorable for therapeutic use.

Another study reported the isolation of seven derivatives of pentacyclic triterpene acids from the bark of *A. viridis* in Serbia, and pentacyclic triterpenoids with a C-27 hydroxymethyl group have been discovered in the genus *Alnus* for the first time (Novakovic et al., 2017). These compounds were cytotoxic toward various cancer cells and were selected for an in silico investigation as potential inhibitors of topoisomerases I and IIα.

9.3 Genus *Euphorbia*

The genus *Euphorbia* (spurge) belongs to the Euphorbiaceae family and consists of more than 2000 species of annual, biennial, or perennial flowering plants, making it one of the biggest and most diverse genera of the entire plant kingdom. Phytochemical studies have shown that plants belonging to this genus contain a wide range of secondary metabolites, including terpenes, glycerols, steroids, cerebrosides, and phenolic compounds that possess antiproliferative, cytotoxic,

antiviral, antimicrobial, anticancer, and antiinflammatory activities (Shi, Su, & Kiyota, 2008; Vasas & Hohmann, 2014). In addition, genus *Euphorbia* contains an abundance of jatrophanes—macrocyclic diterpenes with basic trans-bicyclo [10.3.0]pentadecane structure and a flexible twelve membered ring. Many jatrophanes possess the ability to effectively modulate P-glycoprotein (P-gp) in vitro and consequently reverse the drug-resistant phenotype in cancer cells (Corea et al., 2003a, 2003b; Hohmann et al., 2002), or interact with microtubules (Miglietta, Gabriel, Appendino, & Bocca, 2003). These compounds show cytotoxicity against cancer cells both in single treatments (Hegazy et al., 2010; Lanzotti, Barile, Scambia, & Ferlini, 2015), as well as in combination with anticancer drugs in MDR cells (Pesic et al., 2011).

9.3.1 *Euphorbia dendroides* L

E. dendroides is a perennial deciduous semisucculent bush branching to a height of up to 3 m (Fig. 9.2). It grows in hilly coastal areas of the Mediterranean (mainly Montenegro, Albania, and Greece) (Eichberger, 2003) on a rocky surface, usually in the cracks of the wall (Fig. 9.2). Despite its abundance, *E. dendroides* has not been extensively phytohemically studied. Scientific research on this species has long been limited to its use as a rich source of biomass for fuel production (Sharma, Tiwari, & Behera, 1994). Concerning the biomedical importance of its secondary metabolites, different studies described active substances from *E. dendroides* originating from Greece (Fokialakis, Melliou, Magiatis, Harvala, & Mitaku, 2003), epicuticular waxes from *E. dendroides* (Gülz, Hemmers, Bodden, & Marner, 1987), antioxidant and cytotoxic activity of *E. dendroides* from Egypt (Ibrahim, Mahmoud, & El-Hallouty, 2011), oil (Conti, Marchetti, Usai, & Botteghi, 1988), tocopherols, fatty acids and sterols (Bruni et al., 2004), as well as diterpenes (Corea et al., 2003a, 2003b) from *E. dendroides* originated from Sardinia.

Anticancer properties of two jatrophanes from *E. dendroides* collected in Montenegro were reported in NCI-H460, a sensitive nonsmall cell lung cancer cell line, and its resistant counterpart (NCI-H460/R) (Pesic et al., 2011). The investigated jatrophanes inhibited cancer cell growth, but at the same time displayed no toxicity toward peripheral blood mononuclear cells. In combination treatment, jatrophanes overcame cells' resistance to paclitaxel in a concentration-dependent manner in NCI-H460/R cells. The synergistic effect achieved in combination with paclitaxel as a result of P-gp inhibition, was also observed at the level of cell death induction and cell cycle disturbance—G_2/M arrest (Pesic et al., 2011). In addition, these compounds exerted antiangiogenic properties via a decrease in secretion of the vascular endothelial growth factor (VEGF).

In another study, a strong P-gp inhibiting activity of several new jatrophanes was reported (Jadranin et al., 2013). All jatrophanes included in the study were obtained from the latex of *E. dendroides* collected in Montenegro. Two of the investigated compounds inhibited P-gp more potently than well-known inhibitors R(+)-verapamil and tariquidar in MDR colorectal carcinoma cells DLD1-TxR.

9.3.2 *Euphorbia esula* L

E. esula, or leafy spurge, is a species with a worldwide distribution and in the Balkans it is mostly found in Serbia (Fig. 9.2) (Teuscher & Lindequist, 1994). This plant produces milky, toxic, skin-irritant latex which contains biologically active diterpenes. A phytochemical study was performed on six new jatrophane diterpene polyesters (esulatins H−M), as well as three known compounds including salicinolide and euphosalicin isolated from whole, undried plants of *E. esula* collected near the River Tisza, in Szeged, Hungary (Vasas et al., 2011). Esulatin J showed the most promising biological activity exerting the highest antiproliferative effect against human HeLa, Ishikawa, and MCF-7 cells, as well as L5178 mouse lymphoma cells transfected with the pHa MDR1/A retrovirus DNA. It also displayed potential for reversing drug resistance by inhibiting the function of P-gp transporter.

9.3.3 *Euphorbia nicaeensis* All

E. nicaeensis is a hardy perennial plant characteristic of the Mediterranean region, and also found in Serbia, Romania, and Bulgaria (Fig. 9.2). Root extracts of *E. nicaeensis* All. displayed significant cytotoxicity, while extracts of aerial parts possessed moderate activity (Öksüz, Shieh, Pezzuto, Özhatay, & Cordell, 1993). Epicuticular wax constituents from *E. nicaeensis* (Hemmers & Gidlz, 1986), as well as tetracyclic triterpenoids (Öksüz et al., 1993), glucocerebrosides (Cateni et al., 2003), and glyceroglycolipids (Cateni et al., 2004) exhibited antiinflammatory activity.

Jatrophane diterpenoids, nicaeenin A−G, and euphodendrophanes A−C, F, N, O, Q, and S, isolated from latex of *E. nicaeensis* collected in Serbia showed significant inhibition of P-gp activity in NCI-H460/R and DLD1-TxR, cancer cell lines with MDR phenotype (Krstic et al., 2018). Nicaeenin F and nicaeenin G displayed the highest activity,

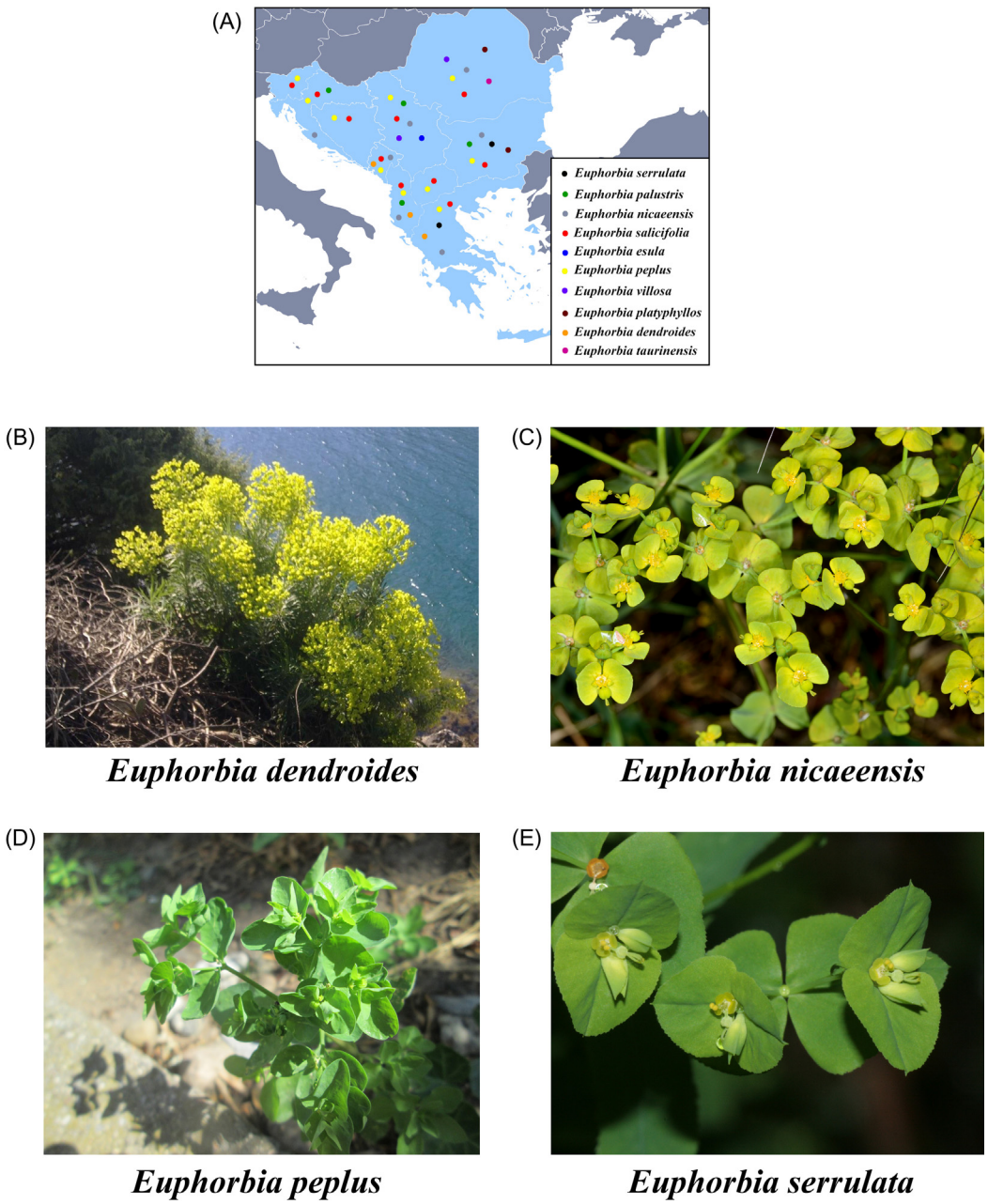


FIGURE 9.2 (A) Distribution area of *Euphorbia serrulata*, *Euphorbia palustris*, *Euphorbia nicaeensis*, *Euphorbia salicifolia*, *Euphorbia esula*, *Euphorbia peplus*, *Euphorbia villosa*, *Euphorbia platyphyllos*, *Euphorbia dendroides*, and *Euphorbia taurinensis* in the Balkans. (B) *Euphorbia dendroides*. (C) *Euphorbia nicaeensis*. (D) *Euphorbia peplus*. (E) *Euphorbia serrulata*.

while nicaeenin G strongly sensitized NCI-H460/R cells to doxorubicin when compared to Dex-verapamil via prolonged P-gp inhibition.

9.3.4 *Euphorbia peplus* L

E. peplus is a weed found in arable lands, forests, rocky and grassy places throughout the Balkans (Fig. 9.2). The latex of *E. peplus* L. was found to contain a number of macrocyclic diterpenes (Rizk, Hammouda, El-Missiry, Radwan, & Evans, 1985), including ingenol mebutate, a compound with very potent anticancer properties (Ramsay et al., 2011).

The sap of this spurge has been shown to be efficient against human nonmelanoma skin cancer in a recent phase I/II clinical study (Ogbourne & Parsons, 2014). Ingenol 3-angelate, another compound found in *E. peplus* was included in a phase II clinical trial for the basal cell carcinoma treatment, squamous cell carcinoma, and intraepidermal carcinoma (Ramsay et al., 2011), and also showed antileukemic properties (Lee et al., 2010). 20-O-acetylingenol 3-angelate was reported to have therapeutic potential for a breast cancer, colon cancer, and melanomas (Mason et al., 2010). Ingenol mebutate isolated from *E. peplus* was used to treat actinic keratosis and in gel form it cleared 71% of treated lesions (Siller, Gebauer, Welburn, Katsamas, & Ogbourne, 2009). A selective activator of protein kinase C, ingenol 3-angelate, was isolated from *E. peplus* and found to induce apoptosis at nanomolar concentrations in myeloid leukemia cell lines and primary acute myeloid leukemia cells (Hampson et al., 2005). In another study, 3-ingenyl angelate application resulted in the clearance of both mouse and human tumors previously established in C57BL/6 or Foxn1(nu) mice (Ogbourne et al., 2004).

9.3.5 *Euphorbia palustris* L

E. palustris, another Euphorbia species commonly distributed in the Balkan region (Serbia, Croatia, Bulgaria, and Albania) (Fig. 9.2), was also reported to contain the potent anticancer compound ingenol mebutate (Beres et al., 2018). The latex extracts of *E. palustris* collected ca. 20 km north of Belgrade, Serbia were found to contain nonpolar metabolites including 24-methylenecycloartanol (Krstic et al., 2016). Previously, 24-methylenecycloartanol was shown to possess cytotoxicity against HeLa, WI-38, and Mel-43 cancer cell lines (Kpoviessi et al., 2008).

9.3.6 *Euphorbia platyphyllos* L

E. platyphyllos, a broad-leaved spurge, is a glabrous or pubescent annual plant primarily distributed in the south of Europe. In the Balkans it is commonly found in the eastern parts including Bulgaria and Romania (Fig. 9.2). The crude extracts of *E. platyphyllos* were good antioxidants showing significant DPPH scavenging activity, as well as cytotoxicity in MCF-7 human breast cancer cells (Aslantürk & Çelik, 2013). Significant DNA damage and apoptosis were also induced which indicates the potential of this plant as an anticancer agent.

9.3.7 *Euphorbia salicifolia* Host

E. salicifolia, is a perennial herb found all over Central Europe and the Balkans (Fig. 9.2) (Tutin et al., 1968). Euphosalicin, a new diterpene polyester obtained from the dichloromethane extract of the whole plant of *E. salicifolia* collected in Hungary, showed activity higher than that of verapamil in reversing drug resistance in mouse lymphoma cells (Hohmann, Evanics, Dombi, & Szabó, 2001). Salicifoline also displayed a prominent effect on reversing MDR in the L5178 mouse T cell lymphoma cell line (Hohmann, Evanics, Dombi, Molnar, & Szabó, 2001).

9.3.8 *Euphorbia serrulata* Thuill

E. serrulata is an annual herb growing in central, western, and southern parts of Europe (Tutin, Heywood, Burges, & Valentine, 1976). In the Balkans it is distributed throughout Bulgaria and Greece (Fig. 9.2). Jatrophane diterpenes isolated from the hexane-soluble extract of the fresh whole plants (Hohmann et al., 2000, 2002) were shown to exhibit MDR-reversing activity in mouse lymphoma cells via prominent P-gp inhibitory effect, moderate cytotoxic activity in Vero cells, and antiviral properties against herpes simplex virus type 2 (Hohmann et al., 2002; Mucsi, Molnar, Hohmann, & Rédei, 2001). A study reported isolation of macrocyclic diterpenes with jatrophane or lathyrane skeletons from methanol extracts of *E. serrulata* collected in Hungary (Engi, Vasas, Redei, Molnar, & Hohmann, 2007). A selected diterpene was evaluated for MDR-reversing activity in human colon cancer cells in vitro, and displayed noteworthy synergistic interaction in combination with epirubicin. This diterpene also showed potential to induce apoptosis in the human cervical adenocarcinoma cell line and human *MDR1* gene-transfected mouse lymphoma cells.

9.3.9 *Euphorbia taurinensis* All

E. taurinensis is a glabrous annual plant found in Central and Southern Europe (Tutin et al., 1968). In the Balkans it generally grows in Romania (Fig. 9.2). Segetane, jatrophane, and ingenane diterpenes isolated from the methanol extract of

E. taurinensis were evaluated for cytotoxic and MDR-reversing action against L5178Y mouse T cell lymphoma cells and the L5178Y human ABCB1-transfected subline (Rédei et al., 2018). Ingenane diterpenes demonstrated a notable cytotoxicity in investigated cell lines, as well as a significant effect on modulating MDR phenotype.

9.3.10 *Euphorbia villosa* W. et K

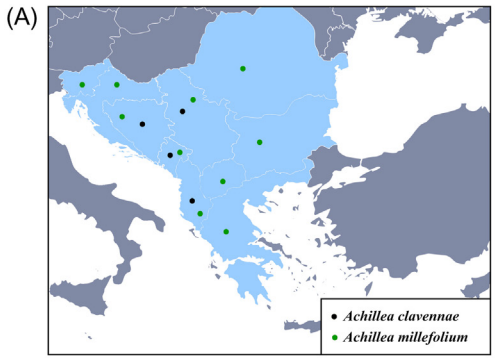
E. villosa is densely tufted perennial herb with glabrous or pilose stems mostly growing in damp shady areas in eastern and southern parts of Europe, but can be also found in France and the British Isles (Pearman, 2007). In the Balkans it is found in Romania and Serbia (Fig. 9.2). A diterpene isolated from the lipophilic phase of *E. villosa* methanol extracts showed a notable additive antiproliferative effect in combination with epirubicin in the human colon cancer COLO320 cell line (Engi et al., 2007). This compound was also found to be a very strong P-gp inhibitor in the investigated MDR colon cancer cells.

9.4 Genus *Achillea*

The genus *Achillea* is a member of the family Asteraceae and comprises around 100 species growing in Europe, mainly the mountainous regions of the Mediterranean, and the temperate areas of Asia (Gajic, 1975).

9.4.1 *Achillea clavennae* L

A. clavennae (silvery yarrow) grows on the mountains in the West Balkans (mainly Bosnia and Herzegovina, Serbia, Montenegro, and Albania) at the altitude of 1500−2500 m (Fig. 9.3). Guaianolides, bisabolenes, and flavonoids, as the main constituents of aerial parts of *A. clavennae* collected in the Komovi Mountain in Montenegro, showed a remarkably high cytotoxic activity in HeLa, K562, and Fem-X human cancer cell lines, particularly the guaianolides 9α-acetoxyartecanin and apressin (Trifunovic et al., 2006). In another study, three new sesquiterpene lactones, 9α-acetoxycanin, sintenin, and oleanolic acid, were investigated for their cytotoxicity in human U251 and rat C6 glioma cell



Achillea clavennae

Achillea millefolium

FIGURE 9.3 (A) Distribution area of *Achillea clavennae* and *Achillea millefolium* in the Balkans. (B) *Achillea clavennae*. (C) *Achillea millefolium*.

lines (Trifunovic et al., 2014). A newly isolated iso-seco-guaianolide displayed strong cytotoxicity due to apoptosis triggering and with efficacy comparable to that of cisplatin and apressin (Valant-Vetschera & Wollenweber, 2001).

9.4.2 *Achillea millefolium* L

A. millefolium is a flowering, aromatic herb native to Europe, North America, Asia, and Australia, and is distributed throughout the Balkan Peninsula (Fig. 9.3). The extracts of *A. millefolium* contain biologically active compounds including asparagin, sterols, isovaleric acid, salicylic acid, flavonoids, tannins, and coumarone (Amini Navaie et al., 2015). The aqueous, ethanolic, and methanolic extracts of the *A. millefolium* flowers and leaves were reported to have antioxidant and antiproliferative properties in the human breast cancer MCF-7 cell line (Amini Navaie et al., 2015).

9.5 Other genera

9.5.1 Genus *Sideritis* L

The genus *Sideritis* (ironwort) belongs to the Lamiaceae family and its members are mainly found in the Mediterranean, and within the Balkans in Albania, Bulgaria, and Greece (Loğoğlu, Arslan, Öktemer, & Şakõyan, 2006). This genus includes annual and perennial herbaceous plants and small shrubs commonly growing in sunny areas on rocky slopes and pastures, from the sea level to more than 3000 m (Abeshi et al., 2017; Davis, Tan, & Mill, 1988; Ramos, Sosa, & de Paz, 1994). The species of this genus are rich in flavonoids, terpenes, and essential oils with prominent pharmacological effects including antiinflammatory (Moroney, Alcaraz, Forder, Carey, & Hoult, 1988), antimicrobial (Dulger, Gonuz, & Aysel, 2006; Dulger, Gonuz, & Bican, 2005), antioxidant (Armata, Gabrieli, Termentzi, Zervou, & Kokkalou, 2008; Hernández-Pérez, Sánchez-Mateo, Montalbetti-Moreno, & Rabanal, 2004), analgesic (Aboutabl et al., 2002), antiulcerogenic (Alcaraz & Tordera, 1988), anticancer (Demirtas, Sahin, Ayhan, Tekin, & Telci, 2009; Kassi et al., 2004), and antiviral activities. A study reported that methanol extract from *Sideritis libanotica* ssp. *linearis* displayed antiproliferative action against human C6, Vero, and HeLa cell lines (Demirtas et al., 2009). Aqueous extracts of *Sideritis euboea* and *Sideritis clandestina* induce osteoblast differentiation in KS483 cells and suppress growth in breast cancer cells (Kassi et al., 2004).

Diethyl ether and ethyl acetate extracts of *S. scardica*, an endemic plant from the Balkans, showed significant cytotoxicity against the rat glioma C6 cells but were not cytotoxic in rat astrocytes in primary culture (Jeremic et al., 2013). Diethyl ether extract triggered apoptosis and autophagy, while ethyl acetate extract induced G_2/M cell cycle arrest and autophagy. The observed effects of *S. scardica* extracts were at least partly mediated by flavonoid components, apigenin and luteolin.

9.5.2 Genus *Laserpitium* L

The genus *Laserpitium* L. belongs to the Apiaceae family and includes aromatic perennial species distributed in the mountainous regions of Central and Southern Europe (Tutin et al., 1968). There are species of this genus that are endemic to the Balkans: *L. ochridanum* Micevski is found in North Macedonia (Micevski, 1993); and *L. zernyi* Hayek is found in Serbia, North Macedonia, Albania, and Greece (Micevski, 1993). Analysis of the chemical composition of extracts from the *Laserpitium* species revealed the presence of sesquiterpene lactones that nearly entirely belong to the class of guaianolides of the slovanolide type (Stefanovic, Milosavljevic, & Bulatovic, 1999). Recently, five guaianolides from the class of slovanolides, and two lactones were isolated from chloroform extracts of the underground parts of these two endemic species and further investigated for their chemical and biological properties (Popovic et al., 2013). Derivatives of slovanolide and silerolide, a phenylpropanoid latifolone and six sesquiterpene lactones, were also isolated from the extracts of *L. zernyi* and *L. ochridanum*. The extracts displayed moderate cytotoxicity against two human breast cancer cell lines, MCF-7/6 and MCF-7/AZ. The extract of *L. ochridanum* was the most efficient in both investigated cell lines, while the compound with the strongest cytotoxic activity in MCF-7/6 cells was a slovanolide derivative with an additional double bond in the lactone ring.

9.5.3 Genus *Digitalis* L

The genus *Digitalis* (foxglove) includes about 20 species of herbaceous perennials, biennials, and shrubs belonging to the Plantaginaceae family and is distributed in central and western regions of Europe with some endemics in the eastern Mediterranean (Bräuchler, Meimberg, & Heubl, 2004). In the Balkans, the most common species of this genus include

Digitalis viridiflora (mainly found in the south of the Balkan Peninsula to the Rhodope Mountains in southern Bulgaria and Greece), *Digitalis grandiflora*, *Digitalis ferruginea* subsp. *ferruginea*, and *Digitalis lanata* subsp. *lanata*, found on the entire Balkan Peninsula, and subsp. *leucophaea* locally distributed in Greece (Bräuchler et al., 2004). Biologically active compounds of foxglove include flavonoids, anthraquinones, cardenolides, and phenylethanoid glycosides (Ganapaty et al., 2003). *Digitalis purpurea* and *D. lanata* are good source of cardiac glycosides (cardenolides) which are used for treating cardiac insufficiency (Fuerstenwerth, 2014; Mijatovic et al., 2007; Navarro et al., 2000). Cardenolides have also been exploited in chemotherapy of prostate and breast cancer lines (Newman, Yang, Pawlus, & Block, 2008). Species of the *Digitalis* genus are also reported to possess emetic, antioxidant cytotoxic, antiinflammatory, antiviral, antibacterial, and antifungal effects (Benli, Yigit, Geven, Guney, & Bingol, 2009; Jin, Jin, Shin, Hong, & Woo, 2011; López-Lázaro et al., 2003; Oh et al., 2005; Orhan, Deliorman-Orhan, & Özçelik, 2009; Warr, Thompson, & Kent, 1992). In a recent study aqueous extracts of the roots and aerial parts of *Digitalis davisiana* Heywood, *D. viridiflora* Lindley, and *D. grandiflora* Miller were investigated in human HEp-2 and HepG2 cancer cell lines (Kutluay & Saracoglu, 2018). The extracts of aerial parts displayed higher cytotoxicity compared to roots and were further tested against human cancer HeLa cells and normal rat cell line 3Y1 and all three extracts exhibited selective cytotoxicity toward HeLa cells.

9.5.4 Genus *Micromeria* Benth

The genus *Micromeria* (Lamiaceae) includes worldwide distributed polymorphic flowering herbs, subshrubs, and shrubs predominantly growing in rocky ground (Slavkovska et al., 2005). In the Balkans it grows throughout the Mediterranean region. Endemic to the southern Carpathian region, *Micromeria pulegium* is a perennial herb mainly found in Romania and Serbia, at an altitude of 1000−1200 m (Haveric, Hindija Čakar, Hadzic, & Haveric, 2018). Other species endemic to the Balkan Peninsula include *M. dalmatica* Benth. (Karousou, Hanlidou, & Lazari, 2012), and *M. fruticosa* Druce (Telci & Ceylan, 2007). Plants belonging to the *Micromeria* genus are rich in essential oils such as pulegone, isomenthone, and menthone (Duru, Öztürk, Uğur, & Ceylan, 2004; Güllüce et al., 2004; Karousou et al., 2012; Radulović & Blagojević, 2012; Slavkovska et al., 2005; Šavikin et al., 2010; Telci & Ceylan, 2007; Vladimir-Knežević, Blažević, & Kaloðera, 2001). The cytotoxic and genotoxic potential of *M. pulegium* aqueous leaf extract was tested in human normal lymphocytes and melanoma GR-M cells (Haveric et al., 2018). The extracts showed no genotoxic effects on human lymphocytes in low concentrations and significantly reduced cell viability in the GR-M melanoma cell line. *M. fruticosa* (L.) Druce ssp. *serpyllifolia* (Bieb) PH Davis, also showed antiproliferative potential in human glioblastoma U87 MG cells (Koc, Ozdemir, Kizilkaya, Sengul, & Turkez, 2017).

9.5.5 Genus *Nepeta* L

The genus *Nepeta* (catnip) includes herbaceous perennial and annual plants in the family Lamiaceae distributed in Central and Southern Europe, West, Central, and Southern Asia, Africa, and North America (Bram, 1974). The plants belonging to this genus are found throughout the Balkan Peninsula. *Nepeta* species are the source of compounds with numerous pharmacological effects, including antiinflammatory (Miceli et al., 2005), analgesic (Aydin, Demir, Öztürk, & Başer, 1999), cytotoxic (Rigano et al., 2011), antiviral (Bedoya, Palomino, Abad, Bermejo, & Alcami, 2002), and antimicrobial effects (Nestorović et al., 2010). Phytochemical studies have revealed that pharmacological activities of plants of the genus *Nepeta* are attributed to secondary metabolites such as phenolics, monoterpenes, diterpenes, and triterpenes, among which nepetalactones are the most frequent (Mišić et al., 2015) and show various biological activities (Liblikas et al., 2005). *N. rtanjensis*, *Nepata sibirica*, and *Nepata nervosa* were shown to be rich in derivatives of hydroxybenzoic and hydroxycinnamic acids (Mišić et al., 2015). Phenolic acids isolated from the *Nepeta* species have been shown to have antioxidant properties (Miceli et al., 2005; Tepe, Daferera, Tepe, Polissiou, & Sokmen, 2007; Yazici, Ozmen, Celikoglu, Ozcelik, & Genc, 2012) and a positive relationship between the amount of total phenolic compounds and antioxidant activity in *Nepata flavida* has been established (Tepe et al., 2007). The methanol extracts of *N. rtanjensis* Diklić & Milojević, an endemic and critically endangered perennial from Serbia (Chalchat, Gorunovic, Petrovic, & Maksimovic, 2000; Skorić et al., 2017), have also been shown to possess strong antioxidant properties (Nestorović Živković et al., 2018). The essential oil of *N. rtanjensis* Diklić & Milojević, rich in *trans*,*cis*-nepetalactone, exhibited significant cytotoxic activity after 72 h of treatment in HeLa, K562, A549, LS-174, and MDA-MB-231 cancer cell lines and was not as potent when tested in normal cell line MRC-5 (Skorić et al., 2017). The application of the essential oil triggered apoptosis in HeLa cells,

confirmed by upregulation of Bax and p53, and downregulation of Bcl-2, and Skp2 genes, highlighting the potential of *N. rtanjensis* essential oil for anticancer treatment.

9.5.6 Genus *Teucrium* L

The genus *Teucrium* (germander) belongs to the family Lamiaceae and consists of more than 300 mostly perennial plants with worldwide distribution (Amirghofran, Zand, Javidnia, & Miri, 2010) but with a number of species endemic to The Mediterranean (Kästner, 1989), and nine species native to the central and west parts of the Balkans (Stankovic, Mitrović, Matić, Topuzovic, & Stamenkovic, 2015). The *Teucrium* species are a source of numerous pharmacologically active compounds including phenols, sterols, terpenoids, diterpenoids, flavonoids, and iridoids (Bahramikia & Yazdanparast, 2012; Eskandry et al., 2007; Rajabalian, 2008). Diterpenoids and methanolic extracts of *Teucrium polium* chemosensitized melanoma Skmel-3, osteosarcoma Saos-2, colon carcinoma SW480, breast carcinoma MCF-7, bladder carcinoma EL, epidermoid carcinoma A431, and oral cavity epidermal KB cells to vincristine, vinblastine, and doxorubicin (Rajabalian, 2008). Numerous other reports have also demonstrated the anticancer effects of *Teucrium* species on human cancer cell lines including breast adenocarcinoma MCF-7, MDA-MB-361 and MDA-MB-453 (Abu-Dahab & Afifi, 2007; Kundaković, Milenković, Stanojković, Juranić, & Lakuscaron, 2011; Talib & Mahasneh, 2010), cervix epitheloid carcinoma, Burkitt's lymphoma (Raji), chronic myelogenous (K562) and T cell leukemia (Jurkat) (Amirghofran et al., 2010), colon carcinoma Caco-2 and HCT-116 (Menichini et al., 2009; Stankovic et al., 2011), glioblastoma multiforme REYF-1 (Eskandry et al., 2007), hepatoblastoma HepG2 (Pacifico et al., 2012), lung carcinoma COR-L23 (Menichini et al., 2009), nonsmall cell lung cancer H322 and A549 (Haïdara, Alachkar, & Al Moustafa, 2011; Pacifico et al., 2012), melanoma C32 (Menichini et al., 2009), prostate carcinoma DU145 and PC3 (Kandouz et al., 2010).

The extracts of seven *Teucrium* species (*T. scordioides*, *T. scordium*, *T. chamaedrys*, *T. polium*, *T. montanum*, *T. arduini*, and *T. botrys*) were investigated for cytotoxicity against human cervix adenocarcinoma HeLa cells, melanoma Fem-x cells, chronic myelogenous leukemia K562 cells, and breast adenocarcinoma MDA-MB-361 cells (Stankovic et al., 2015). All extracts were effective against HeLa, but showed low cytotoxicity on the MDA-MB-361 cell line. In K562 cells, *T. scordioides*, *T. montanum*, and *T. botrys* displayed the most notable effect. Moreover, *T. scordioides* and *T. montanum* extracts exhibited low cytotoxicity when tested on healthy resting and phytohemagglutinin-stimulated peripheral blood mononuclear cells, making these species a promising natural source of effective biological compounds. The methanolic extracts of *T. chamaedrys* and *T. arduini* are also characterized with the highest abundance of phenolic compounds that showed the strongest cytotoxicity in human colon carcinoma HCT-116 cell line (Stankovic et al., 2011).

9.5.7 Genus *Salvia* L

The genus *Salvia* (sage) is the largest one in the Lamiaceae family and consists of almost 1000 species of shrubs, herbaceous perennials, and annuals. Dalmatian sage, *Salvia officinalis* L., a perennial, evergreen subshrub, is native to the Mediterranean (Grdiša et al., 2015). Its indigenous distribution in the Balkans includes Croatia, Bosnia and Herzegovina, Montenegro, Albania (Grdiša et al., 2015), northern Greece (Karousou, Hanlidou, & Kokkini, 2000), and southern Serbia (Janković, 1982), but it is also cultivated in Bulgaria and Romania (Raal, Orav, & Arak, 2007). *S. officinalis* L. is a rich source of biologically active compounds with antidiabetic (Eidi & Eidi, 2009; Hamidpour, Hamidpour, Hamidpour, & Shahlari, 2014; Swanston-Flatt, Flatt, Day, & Bailey, 1991), antioxidative (Nickavar, Kamalinejad, & Izadpanah, 2007; Yadav & Mukundan, 2011), gastroprotective (Mayer et al., 2009), antiinflammatory (Baricevic & Bartol, 2000; Ninomiya et al., 2004), antiobesity (Ninomiya et al., 2004), antimutagen (Patenkovic, Stamenkovic-Radak, Banjanac, & Andjelkovic, 2009), antiviral (Schnitzler, Nolkemper, Stintzing, & Reichling, 2008; Smidling, Mitic-Culafic, Vukovic-Gacic, Simic, & Knezevic-Vukcevic, 2008; Tada, Okuno, Chiba, Ohnishi, & Yoshii, 1994), fungicidal, and bactericidal properties (Bouaziz, Yangui, Sayadi, & Dhouib, 2009; Delamare, Moschen-Pistorello, Artico, Atti-Serafini, & Echeverrigaray, 2007; Khalil & Li, 2011; Pinto, Salgueiro, Cavaleiro, Palmeira, & Gonçalves, 2007). *S. officinalis* L. also possesses significant anticancer properties (Hamidpour et al., 2014; Itani et al., 2008; Jedinak, Mučková, Košťálová, Maliar, & Mašterová, 2006). It was reported that urosolic acid *from S. officinalis* inhibited angiogenesis, invasion, and metastasis in the B16 melanoma cell line (Jedinak et al., 2006). The extract of *S. officinalis* inhibited proliferation, migration, and angiogenesis in human umbilical vein endothelial cells (HUVECs), mouse fibrosarcoma (Wehi), and human myelogenous leukemia K562 cells (Keshavarz, Bidmeshkipour, Mostafaei, Mansouri, & Mohammadi, 2011). Diterpenoids isolated from the *S. officinalis* root displayed strong cytotoxicity and

induced DNA damage in human colon carcinoma Caco-2 and hepatoma HepG2 cells (Slameňová et al., 2004). The essential oil of *S. officinalis*, containing α-humulene and *trans*-caryophyllene, showed cytotoxic activity in breast cancer MCF-7, colon cancer HCT-116, and murine macrophage RAW264.7 cell lines (El Hadri et al., 2010).

9.5.8 Genus *Helichrysum* Mill

The genus *Helichrysum* belongs to the family Asteraceae and consists of approximately 600 species of flowering plants. It is distributed in Europe, Asia, Africa, Madagascar, and the Australian region (Galbany-Casals et al., 2014). The *Helichrysum* species native to the Balkan Peninsula include *Helichrysum plicatum* DC. Prodr. (found in Serbia, North Macedonia, Albania, and Greece, but also in the Anatolian Peninsula and Iran) (Bayer, 2007), and *H. italicum*, which is commonly distributed in the Mediterranean (Galbany-Casals, Blanco-Moreno, Garcia-Jacas, Breitwieser, & Smissen, 2011). The extracts of many of these plants are sources of numerous biologically active phytochemicals, including flavonoids, acetophenones, phloroglucinols, pyrones, diterpenes, and sesquiterpenes with significant antibacterial, antiviral, antifungal, antioxidant, antiinflammatory, and antidiabetic properties (Aiyegoro & Okoh, 2010; Angioni et al., 2003; Appendino et al., 2007; Aslan, Orhan, Orhan, Sezik, & Yesilada, 2007; Lourens, Viljoen, & Van Heerden, 2008; Meyer, Afolayan, Taylor, & Engelbrecht, 1996; Nostro et al., 2001; Sala, Recio, Giner, Máñez, & Ríos, 2001; Süzgeç, Meriçli, Houghton, & Çubukçu, 2005). This genus is also a significant source of compounds with anticancer potential (Afoulous et al., 2011; Bauer et al., 2011; Guinoiseau et al., 2013; Kucukoglu, Ozturk, Kamataki, & Topcu, 2006; Yagura et al., 2008).

Helichrysum zivojinii Černjavski & Soška is an endemic plant species growing in North Macedonia. The extracts of *H. zivojinii* exerted selective cytotoxicity against HeLa, Fem-x, and K562 human cancer cell lines and healthy immunocompetent human peripheral blood mononuclear cells (PBMC), while the effect on unstimulated PBMC was not as distinct (Matić et al., 2013). The extracts triggered both intrinsic and extrinsic apoptotic signaling pathways in HeLa cells.

Two structurally distinct chalcone dimers, isolated from the aerial parts of *H. zivojinii* Černjavski & Soška, were investigated for their anticancer potential in the human cancer cell line NCI-H460 and its resistant variant NCI-H460/R. Tomoroside A inhibited *TOPO IIα* and *HIF-1α* expression and enhanced the anticancer action of doxorubicin, while tomoroside B synergized with tipifarnib, increased *HIF-1α* expression, modulated redox status, and acted as an antioxidant (Aljancic et al., 2014).

Helichrysum arenarium (L.) Moench, is a herbaceous perennial plant broadly distributed in Europe and Asia, and is mainly present in the northern parts of the Balkan Peninsula (Greuter, 2006). Naringenin, one of the most abundant flavonoids found in *H. arenarium*, was described as an inhibitor of Two-Pore Channel 2 (TPC2)-mediated signaling, that is involved in pathological conditions such as melanoma, Ebola virus infection, and Parkinson's disease (Pafumi et al., 2017). Naringenin also showed anticancer potential in human placental choriocarcinoma cells by reactive oxygen species generation and MAPK pathway activation (Park, Lim, Bazer, & Song, 2018).

9.6 Division Marchantiophyta

The division Marchantiophyta (liverworts) belongs to bryophytes, a group of nonvascular plants with approximately 24,000 species, that are taxonomically placed between algae and pteridophytes (Asakawa, Ludwiczuk, & Hashimoto, 2013). This group also includes two other divisions, Bryophyta (mosses), and Anthocerotophyta (hornworts), and its members usually grow on wet soil or rock, tree trunks, rivers, and lakes (Asakawa et al., 2013).

9.6.1 *Marchantia polymorpha* L

M. polymorpha (Marchantiaceae) is a large thalloid liverwort with a worldwide distribution. In Europe, this liverwort is most common in humid and endolithic areas of Iceland (Jóhannsson, 2002) but can also be found throughout the entire Balkan region including Serbia (Fig. 9.4) (Sabovljević et al., 2017). Macrocyclic bisbibenzyls, a class of compounds exclusively produced by liverworts, possess numerous biological activities, such as cytotoxic, antibacterial, antifungal, and antioxidative activities (Jensen, Omarsdottir, Thorsteinsdottir, Ogmundsdottir, & Olafsdottir, 2012).

Marchantin A, a cyclic bisbibenzyl isolated from *M. polymorpha* and other liverworts, has been found to exhibit various biological and pharmacological properties (Asakawa, Ludwiczuk, & Nagashima, 2009; Huang et al., 2010; Iwai et al., 2011; Keserű & Nogradi, 1995). This compound has been reported to display cytotoxicity in human HeLa subline KB cancer cells, P-388 murine leukemia (Keserű & Nogradi, 1995), MCF-7 breast adenocarcinoma (Huang et al., 2010), and antimicrotubule activity in HeLa cells (Jian et al., 2009). Marchantin A from *M. polymorpha* and

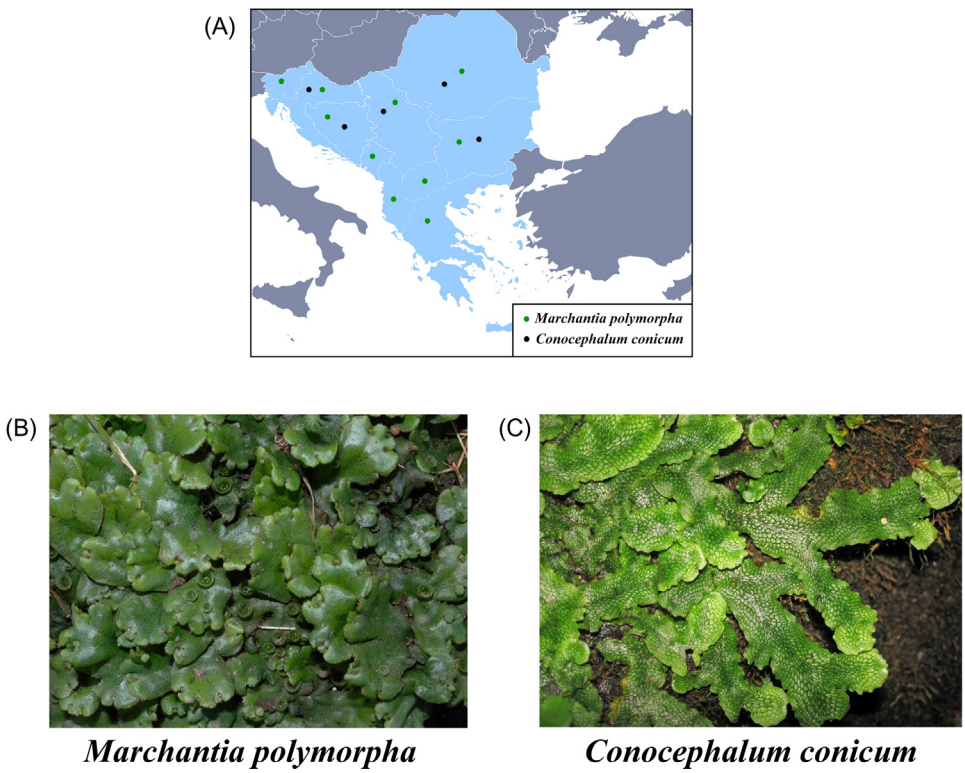


FIGURE 9.4 (A) Distribution area of *Marchantia polymorpha* and *Conocephalum conicum* in the Balkans. (B) *Marchantia polymorpha*. (C) *Conocephalum conicum*.

Marchantia tosana exerted cytotoxicity against the KB cells (Asakawa, Toyota, Taira, & Takemoto, 1982), induced cell growth inhibition and apoptosis in human MCF-7 breast cancer cell line, increased expression of p21 and p27 genes, and reduced cyclin B1 and D1 expression (Huang et al., 2010). Marchantin A reduced cell viability in breast cancer cell lines A256 and MCF-7 and T47D, and showed considerable synergy in combination with the Aurora-A kinase inhibitor MLN8237 and strong antimicrotubular effect (Jensen et al., 2012).

Marchantin C, another compound that can be found in *M. polymorpha* (Asakawa, Okada, & Perold, 1988; Asakawa, Tori, Takikawa, Krishnamurty, & Kar, 1987), exerted cytotoxic effect against the P-388 leukemia cell line (Scher, Burgess, Lorimer, & Perry, 2002) and promoted apoptosis in human glioma A172 cells (Shi, Liao et al., 2008). Marchantin C also arrested the cell cycle in G_2/M phase in A172 and Hela cells and decreased the number of microtubules (Shi et al., 2009). Furthermore, marchantin C reduced the levels of matrix metalloproteinase 2 and inhibited the migration in T98G and U87 glioma cells (Shen et al., 2010), while it was effective in inhibiting P-gp activity in vincristine-resistant KB/VCR cells (Xi et al., 2010).

Cyclic bisbibenzyl plagiochin E, isolated from *M. polymorpha*, decreased the antiapoptotic protein Bcl-2 and increased proapoptotic Bax expression, PARP cleavage, and caspase-3 activity in chemoresistant prostate cancer PC3 cells (Xu et al., 2010).

9.6.2 *Conocephalum conicum* (L.) Dum

C. conicum (Conocephalaceae), a thalloid liverwort which grows on wet rock or soil, is one of the most widespread liverwort species in the world. In the Balkans it is distributed in Croatia, Bosnia and Herzegovina, Serbia, Romania, and Bulgaria (Fig. 9.4). *C. conicum* is a source of pharmacologically active compounds with antibiotic properties (Asakawa, 1995; Castaldo-Cobianchi, Giordano, Basile, & Violante, 1988; Singh, Singh, Nath, Sahu, & Singh Rawat, 2011). Anticancer activity of its component—a monoterpene ester, 2α,5β-dihydroxybornane-2-cinnamate—has also been reported in human liver cancer HepG2 cells (Lu, Fan, Ji, & Lou, 2006). Importantly, *C. conicum* is a source of apigenin and luteolin (Markham, Porter, Mues, Zinsmeister, & Brehm, 1976), flavonoids widely investigated for their anticancer activities (Seelinger, Merfort, Wolfle, & Schempp, 2008; Shankar, Goel, Gupta, & Gupta, 2017; Tuorkey, 2016; Yan, Qi, Li, Zhan, & Shao, 2017).

9.7 Traditional medicinal uses of plants from the Balkans

Plants of the genus *Alnus* are widely recognized for their traditional medical values. They are used as alteratives (blood cleansing agents), astringents, purgatives, emetics, antipyretics, hemostatics, antiparasitics, antihelmintics, for stimulation of mammary glands, as well as for treating various diseases, including tumors (Hylton, Holtom, & Braun, 1979). The extracts from the *A. glutinosa* bark are known for their alterative, astringent, purgative, and antipyretic properties and were used to treat inflammation in the oral cavity (Hylton et al., 1979). The extracts from the inner bark of this plant were used against lice and various skin problems such as scabies (Hylton et al., 1979; Launert, 1981; Lust, 1974). The bark of *A. glutinosa* was also used to treat swellings, inflammation, and rheumatism (Hylton et al., 1979), as an astringent, emetic, and hemostatic, as well as to treat nausea and pharyngitis (Middleton et al., 2010).

The latex of *E. dendroides* was used in the past to treat warts and verrucas, and as a strong purgative or an emetic, while the root powder was used to cure rheumatic pains (Afferni, 2012). In Asia, the plant was used against snake bites and aquatic insects (Afferni, 2012). The extracts of *E. esula* have been extensively used in traditional medicine for treating cancer, swellings, and warts (Hartwell, 1970). Traditionally, *E. peplus* has a long history of use for a variety of disorders. The sap has been used as a purgative and for treatment of warts, waxy growths, corns, catarrh, asthma, as well as skin, stomach, liver, and uterus cancers (Hartwell, 1970; Rizk et al., 1985). The latex and other parts of *E. platyphyllos*, have traditionally been used in medicine for treating warts, wens, and hangnails (Hartwell, 1970).

The members of the *Achillea* genus, particularly their aerial parts, have long been utilized in traditional medicine. *A. clavennae* has been used for the preparation of remedies with cholagogue, digestive, and anthelmintic effects since the 17th century (Maggioni, 1953), and also possesses antiinflammatory, spasmolytic, hemostatic, antibacterial, and antifungal properties (Bezić, Skočibušić, Dunkić, & Radonić, 2003; Chalchat, Gorunovic, & Petrovic, 1999; Duke, Bogenschutz-Godwin, duCellier, & Duke, 1987; Gomez, Saenz, Garcia, & Fernandez, 1999; Honda et al., 1996; Simic, Palic, Vajs, Milosavljevic, & Djokovic, 2002). In Croatia, *A. clavennae* has been used against diarrhea, abdominal pain, fever, cold, and influenza. *A. millefolium* essential oil has been used in folk medicine for its antibacterial, astringent, diuretic, digestive, antiinflammatory, anticancer, and antiallergic effects (Cekic, Kilcar, Muftuler, Unak, & Medine, 2012; Sant'Anna et al., 2009).

The *Sideritis* species have traditionally been used to aid digestion, boost the immune system and against the flu, sinus congestion, allergies, to relieve pain, and mild anxiety (Abeshi et al., 2017). Mountain tea, *S. scardica*, an endemic plant of the Balkan Peninsula, has been a traditional natural remedy used in folk medicine for centuries for treating bronchitis, rheumatic, and gastric disorders (Jeremic et al., 2013).

Several widely distributed *Laserpitium* species have a long history of application in traditional medicine. For instance, *Laserpitium siler* roots and rhizomes were used against toothache, while the *Laserpitium latifolium* underground parts helped in treating gastrointestinal disorders, heart and liver dysfunctions, pulmonary tuberculosis, rheumatism, dermatomycoses, and were used as emenagogues and diuretics (Hegi, 1912; Kuprevič, 1974; Popovic et al., 2013; Vereskovskii, Kuznetsova, Loznukho, Sokolov, & Osokin, 1992).

The species of genus *Digitalis* have been used as a folk medicine since the 18th century as a diuretic (Navarro et al., 2000) and for treating heart and kidney diseases (Baytop, 1999; Goldman, 2001).

The *Micromeria* species are broadly used as aromatic culinary supplements and also possess medicinal value due to their bioactive secondary metabolites such as terpenoids, phenols, and essential oils. In traditional medicine *Micromeria* species have been used to treat headache, wounds, and heart disorders (Formisano, Oliviero, Rigano, Saab, & Senatore, 2014). They were also reported to have antiinflammatory, antibacterial (Ali-Shtayeh, Al-Nuri, Yaghmour, & Faidi, 1997), and antioxidative effects (Couladis, Tzakou, Verykokidou, & Harvala, 2003; Güllüce et al., 2004). The leaf decoct of *Micromeria* plants is used to treat stomach ache, cold, fever, and wounds (Benomari et al., 2016).

The *Nepeta* species have long been used in traditional medicine as they have antispasmodic, antiasthmatic, diuretic, antitussive, antiseptic, and febrifuge activities (Padure, Badulescu, Burzo, & Mihăiescu, 2006). Catnip tea possesses anticholinergic effects and is used to treat intestinal cramps, flu, fever, and insomnia (Simon, Chadwick, & Craker, 1984).

The species of the *Teucrium* genus have been used since the ancient times as spices, tea, as well as for treatment of various health issues (Stankovic et al., 2015). *T. polium* has a 2000-year-long medicinal application against abdominal pain, indigestion, common cold, diabetes, and urogenital diseases (Bahramikia &Yazdanparast, 2012; Rajabalian, 2008; Said, Khalil, Fulder, & Azaizeh, 2002). In the Balkans, plants most commonly used as traditional remedies include *T. chamaedrys*, *T. montanum*, and *T. scordium* (Kundaković et al., 2011). These plants are used for treating wounds, fever, as cholagogue, antianemic, and antimotility agents (Jarić et al., 2007; Kundaković et al., 2011; Redžić, 2007).

The healing properties of the genus *Salvia* (lat. salvere, meaning to save) have been recognized since the ancient times (Dweck, 2000). Sage tea is a traditional natural remedy for teeth and gums infections, sore throat, as an anticough

medicine, a diuretic, and a wound-healing agent (Panda, 2006). *S. officinalis* has been used for relieving menopause symptoms (Bommer, Klein, & Suter, 2011) and also exhibited favorable effects on memory disorders, depression, cognition, and cerebral ischemia (Eidi, Eidi, & Bahar, 2006; Ferreira, Proença, Serralheiro, & Araujo, 2006; Moss, Rouse, Wesnes, & Moss, 2010; Perry et al., 1999; Tildesley et al., 2005). *S. officinalis* also has application in skin care to treat various skin injuries (Grdiša et al., 2015).

Some species from the genus *Helichrysum* have a worldwide application in traditional medicine as a remedy for wounds, respiratory infections, and gastrointestinal disorders (Lourens et al., 2008; Passalacqua, Guarrera, & De Fine, 2007; Redžić, 2007; Sezik et al., 1997). *H. plicatum* has been traditionally used in Serbia and North Macedonia for treating gastric and hepatic disorders (Kulevanova, Stefova, & Stafilov, 2000), and also possesses antidiabetic, antibacterial, and antioxidant activity (Aslan et al., 2007; Smirnov, Preobrazhenskaia, & Kalashnikov, 1982; Tepe, Sokmen, Akpulat, & Sokmen, 2005). The flowers of *H. arenarium* are traditionally used in European ethnomedicine as a choleretic and cholagogue, as well as a hepatoprotective and detoxifying herbal drug (Pljevljakušić, Bigović, Janković, Jelačić, & Šavikin, 2018).

M. polymorpha is one of the most commonly used liverworts in traditional medicine (Harris, 2008). It has been used worldwide for liver ailments (Hay, 2003; Pant, 1998) and as an ointment to treat skin disorders such as boils, eczema, cuts, bites, wounds, and burns (Ando, 1982; Asakawa et al., 2013; Glime, 1978). The use of this plant for its antipyretic, antidotal, and diuretic activity was also reported (Asakawa et al., 2013).

C. conicum has been known for its antimicrobial, antifungal, antipyretic, antidotal activity, and has been traditionally used to cure cuts, burns, scalds, fractures, swollen tissue, poisonous snake bites, and gallstones (Asakawa, 1999; Asakawa, 2008a, 2008b).

9.8 Conclusions

Combining traditional knowledge with currently available research tools such as combinatorial chemistry, phenotype and genotype-based screenings, molecular and synthetic biology may open new perspectives for anticancer drug discovery from natural sources. However, from a scientific point of view, there are several issues that should be considered if we wish to achieve sustainable harvesting and drug production: (1) access and availability of resources; (2) potential for cultivation and protection; (3) in situ and ex situ conservation; (4) impact of climate change; and (5) standardization of protocols for isolation of active constituents—extracts and pure compounds. Certainly, there are more legal issues that are limiting full access to traditionally used medicinal organisms but they are beyond the scope of this chapter.

Due to the aforementioned matters, remarkable results achieved in preclinical research rarely move on to clinical trials. The lack of interest from capital investors such as national governments and big pharmaceutical companies significantly diminishes the enthusiasm of scientists involved in natural products research. The anticancer potential of natural products from the Balkans is considerable and certainly deserves more attention and efforts from all stakeholders (policy makers, pharmaceutical companies, biotechnological companies, and local communities). The legacy that we will leave to future generations depends on our immediate actions with respect to the sustainable use of nature's treasures from the Balkan Peninsula.

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Chapter 10

Biodiversity of wild fruits with medicinal potential in Serbia

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10.1 Wild fruits

As regular cenobionts of different natural plant communities, wild fruits have an important role in both the phytosociological composition of a community and the numerous interactions between all community members. Wild fruits represent an important element in food chains, sharing a significant part in the feed of small mammals and resident and migratory birds over winter. The edible fruits may be dry or soft; the pericarp of soft fruits is fleshy, either partially (when it contains fleshy and firm parts) or completely (as in the berry). Wild fruits are the preferred group of wild edible plants due to their availability, pleasant taste, and aroma, and also because of their pharmacological properties that have been recognized in different traditional pharmacopoeias. Most edible fruits can be consumed fresh, but they are also frequently dried or preserved in different products (jams, marmalade, compotes, beverages). Some wild fruits are not tasty for consumption, even when not toxic. The unattractive taste or palatability of these fruits is due to their bitterness/astringency, but even these are sometimes considered as potentially edible (after processing) (Etkin, 2000).

10.2 Natural functional food

The nutritional composition of wild fruits is highly rated as it comprises plenty of dietetic fibers, vitamins, minerals, and secondary metabolites. There is evidence that different parts of wild fruits were incorporated in daily nourishment in ancient civilizations, being used for diet, the prevention and curation of health disorders, and for cosmetic preparations (Etkin, 2000). Each edible product of biological origin, in addition to its essential role of providing energy and building molecules, also carries a potential in the different bioactive properties of its minor constituents, that is, bioactive molecules (Ferreira, Morales, & Barros, 2016; Harvey, Edrada-Ebel, & Quinn, 2015). Recognizing the beneficial effects of foods containing bioactive molecules has led to the establishment of the terms "functional food" (food that is regularly consumed and exerts one or more target functions in human physiology, with the potential to improve health) and "nutraceuticals" (physiological effective food/plant ingredients or extracts, not consumed in normal diet but as pharmaceutical products) (Bagchi, 2006; Murray, Pizzorno, & Pizzorno, 2005). Natural food sources are considered as functional food, regardless of whether they are consumed as integral (unmodified) food or processed, with a specific chemical composition (Bagchi, 2006). Current research in the field of functional foods draws substantially from multidisciplinary studies, food science, biotechnology, pharmacology, biochemistry, biomedicine, and related scientific disciplines.

10.3 Preventive nutrition and biomedicine

It is known that a healthy diet, including functional foods, provides numerous benefits to human well-being, that is, maintaining a good general health status, preventing degenerative processes, and even improving some health disorders (Blumenthal, Goldberg, & Brinckmann, 2000). Wild fruits are considered to possess more health-beneficial elements in comparison with related cultivated fruit species. The content of secondary metabolites in wild fruits, usually those with

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strong antioxidant characteristics, is higher because these plants are exposed to numerous biotic interactions in natural habitats and have powerful defense mechanisms against herbivores (Lankadurai, Nagato, & Simpson, 2013). On the other hand, cultivated species, being protected by different plant-protection products, synthesize less of their own defense chemicals; also, being constantly treated with herbicide and pesticide agents, they become less attractive for potential consumers as "healthy food" (Bajić-Ljubičić, Popović, Matić, & Bojović, 2018).

Although preventive nutrition falls into the field of nutrition science, it is also close to biomedicine since it is aimed at preventing or reducing the development of disease and its consequences. Nowadays, diet is considered the key environmental factor in the incidence of many chronic diseases, and much effort has been put into finding new preventive strategies and actions for decreasing disease risks (Di Renzo & De Lorenzo, 2016). Current research in the field of functional food chemistry, bioactive substances, and biomedicine should contribute to the development of these strategies.

10.4 Wild fruits in Serbia

Serbia is located in the southeast Europe, mostly on the Balkan Peninsula (Fig. 10.1). The territory of Serbia is characterized by high genetic, species, and ecosystem diversity, which is the result of the specific climatic, orographic, edaphic, and historical conditions. Among the plant species that inhabit autochthonous plant communities in Serbia, wild fruits represent a specific group of species, highly diverse in terms of taxonomy, ecology, phenology, life forms, and other characteristics. Generally, wild fruits inhabit natural communities, mostly hilly and mountainous mesophilous deciduous forests, and are edible or potentially edible. According to relevant scientific publications, the research interest in the natural occurrence and ecology of wild fruits is evident (Bojović et al., 2014; Matić et al., 2015). In total, 99 wild-fruit species are inventoried in Serbian flora, belonging to 27 genera and 18 families (Lakušić et al., 2005; Mratinić & Kojić, 1998).

FIGURE 10.1 Serbia—map and location.

To focus on studies of these fruit-bearing wild plants, we surveyed peer-reviewed scientific literature using academic internet search engines with access to all major databases in the natural sciences. The material includes articles published between January 1, 2000 and December 31, 2018. Documents used in this study were primarily derived by Scopus; additional searches were performed using Google Scholar, Thompson Reuters, Web of Science, Science Direct, Wiley InterScience, and SpringerLink. All searches were conducted using the Latin and common names of all the wild-fruit species inventoried in Serbian flora as the keywords. From the literature search, articles that were selected for further analysis included studies that focused on the pharmacological and biological effects of all parts of fruit (exocarp, mesocarp, endocarp, and seed). Articles dealing with fruit quality (after storage, different treatment for their preservation, etc.) were not included in this analysis. In total, 797 original research and 61 review articles were analyzed. Microsoft Excel 2010 was used to create a unifying database (reference list is available upon request). The data on wild-fruit species in Serbian flora, their biological/pharmacological activity, and the model system in which this activity was demonstrated are collated in Table 10.1.

10.5 Biologically active natural compounds

Chemical characterization of natural molecules is usually associated with studies of their structural diversity, biological activities, and the biochemical mechanisms of their potential therapeutic role. The interest of pharmacological companies in intensive research in this field dates back to the discovery of antibiotics, and in recent years it has become increasingly relevant in the search for new drugs. The occurrence of a large number of different chemical compounds in biological systems is the subject of consideration of various evolutionary theories, since the concept of natural selection implies that the cost of the synthesis of any organic molecule would have purpose only if it increases fitness (Firn & Jones, 2003). The broad range of different natural chemicals indicates infinite biological and chemical diversity and draws attention to the biotic relationships that are established in natural ecosystems (Berenbaum & Zangerl, 1996). The biological activity of natural products in the light of natural selection does not correspond to the expected applicability for pharmacological purposes. From an evolutionary/ecological perspective, an organic compound found in a taxon would not necessarily exhibit biological activity to another taxon that does not share the same habitat, resources, trophic chain, biotic interactions, etc., and only a small percentage of tested compounds exhibit biological activity of pharmacological importance (Firn, 2003). Even when the molecular structure of the compounds is determined, their activity in other biological systems is not predictable, which leads to intensive testing of the vast number of compounds in in vitro and in vivo studies. Products of natural origin that have confirmed their activity in other biological systems are isolated from plants, mushrooms, animals, and microorganisms (Bidlack, Omaye, Meskin, & Topham, 2000). Phytochemical testing of plants and pharmacological examination of their products is most often indicated by the data on the use of certain species in traditional medicine in different cultures (Popović, Matić, Bojović, Stefanović, & Vidaković, 2016). Considering that among the total number of plant species on Earth (250,000−500,000), 50,000 species are used for medical purposes (Idu, 2013) and only about 5000 species are phytochemically characterized (Rates, 2001), further development in this field is expected in the years to come.

10.6 Biomedical significance of wild fruits

Out of 99 species found in the Serbian flora, 60 have been tested in biomedical studies, and a total of 90 different biological activities have been attributed to their fruits (Table 10.1). Of the wild-fruit flora, the most common are members of the Rosaceae family, and the genera represented with the largest number of species are *Rosa*, *Rubus*, *Prunus*, and *Sorbus* (Fig. 10.2). A variety of fruit types are included in this group of plants (Fig. 10.3).

Biomedical studies are focused on establishing the biological activities of wild fruits in different model systems: in vitro, in vivo, ex vivo assays, cell cultures (animal and human cell lines), in vivo animal models, human volunteer studies, and clinical trials (Fig. 10.4). Due to the large number of determined biological activities, 15 major groups were distinguished (Fig. 10.5), and specific activities within each group are shown in (Fig. 10.6).

10.6.1 Antioxidant and antiradical activity

Being a normal by-product of aerobic cellular metabolism, free radicals are considered as highly reactive and harmful entities that damage biomolecules, which can lead to many serious diseases and generally advance the aging process. Recent studies provide evidence that the most important classes of radical species (reactive oxygen and nitrogen species—ROS and RNS, respectively) are generated in strongly regulated processes and have an important role in cell

TABLE 10.1 Wild fruits species in Serbia. Biological and pharmacological activities listed with number of studies supporting the particular activity of a species; study protocols listed with number of studies regarding the particular model system.

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Amelanchier ovalis* Med.** Snowy mespilus (merala)	Rosaceae	Antibacterial	1	Inhibition of bacterial growth	1
***Berberis vulgaris* L.** Common barberry (šimširika)	Berberidaceae	Antioxidant	10	In vivo rat	6
		Anticancer	3	In vitro assay systems	4
		Hepatoprotective	3	Clinical trial	3
		Antidiabetic	2	Human cell lines	3
		Antiinflammatory	2	In vivo mouse	3
		Cytotoxic	2	In vivo and in vitro mouse	1
		AChE inhibitory	1	In vivo sheep	1
		Anticolitis	1	Inh. of fungal growth	1
		Antifungal	1	Mouse cell line	1
		Antihypertensive	1		
		Antimicrobial	1		
		Antiparasitic	1		
		Antiulcerogenic	1		
		Gastroprotective	1		
		Hypoglycemic	1		
		Nephroprotective	1		
		Neuroprotective	1		
		Proapoptotic	1		
		Sedative	1		
***Castanea sativa* Mill.** Sweet chestnut (pitomi kesten)	Fagaceae	Antioxidant	15	In vitro assay systems	14
		Cytotoxic	2	Human cell lines	3
		Antiproliferative	1		
		Cytoprotective	1		
***Celtis australis* L.** European nettle tree (košćela)	Cannabaceae	Analgesic	1	Antimicrob. activ. ass.	1
		Antifungal	1	In vivo rat and mouse	1
		Antiinflammatory	1		
		Antimicrobial	1		
		Antioxidant	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Cornus mas* L.** Cornelian cherry (dren)	Cornaceae	Antioxidant	36	In vitro assay systems	27
		Antiinflammatory	6	In vivo rat	11
		Antihyperlipidemic	4	In vivo rabbit	5
		Antiatherogenic	3	Human cell lines	4
		Antihyperglycemic	3	In vivo mouse	3
		Hypolipidemic	3	Antimicrob. activ. ass.	1
		Antibacterial	2	Clinical trial	1
		Antiobesity	2	Double-blind study	1
		Antiradical	2	Green monkey Cell l.	1
		Hepatoprotective	2	In vivo hamster	1
		Neuroprotective	2	Inh. of bacterial grow.	1
		Anticancer	1	Toxicological study	1
		Antiepileptic	1		
		Antihypercholesterol	1		
		Antihypertriglycerid	1		
		Antimicrobial	1		
		Antiparasitic	1		
		Antiproliferative	1		
		Antitumor	1		
		Cardioprotective	1		
		Cytoprotective	1		
		Hypocholesterolemic	1		
		Hypotriglyceridemic	1		
		Immunostimulatory	1		
		Nephroprotective	1		
		Ophthalmic protective	1		
		Skin protective	1		
***Cornus sanguinea* L.** Common dogwood (svib)	Cornaceae	Antioxidant	2	In vitro assay systems	2
***Corylus avellana* L.** Common hazel (leska)	Betulaceae	Antioxidant	11	In vitro assay systems	9
		Allergenic activity	4	Human cell lines	4
		Antiatherogenic	2	Human volunteers	3
		Antibacterial	2	Antimicrob. activ. Ass	1

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
		Antiradical	2	In vivo and in vitro mouse	1
		Antiinflammatory	1	Inh. of bacterial grow.	1
		Antimicrobial	1		
		Antiobesity	1		
		Cytotoxic	1		
		Hypolipidemic	1		
		Neuroprotective	1		
		Prebiotic	1		
***Corylus colurna* L.** Turkish hazel (mečja leska)	Betulaceae	–		–	
Cotoneaster integerrimus Medik. Common cotoneaster (dunjarica)	Rosaceae	Antibacterial	1	In vitro assay systems	1
		Antioxidant	1		
Cotoneaster nebrodensis (Guss.) Koch Brickberry cotoneaster (pustenasta dunjarica)	Rosaceae	–		–	
Crataegus heldreichii Boiss. (Heldrajhov glog)	Rosaceae	–		–	
***Crataegus monogyna* Jacq.** Common hawthorn (beli glog)	Rosaceae	Antioxidant	22	In vitro assay systems	15
		Cardioprotective	7	In vivo rat	5
		Antibacterial	5	Human cell lines	3
		Antiproliferative	3	Clinical trial	1
		Analgesic	2	Double-blind study	1
		Antiinflammatory	2	In vivo mouse	1
		Antimicrobial	2	In vivo rabbit	1
		Cytoprotective	2	Inh. of bacterial grow.	1
		Gastroprotective	2	Mouse cell line	1
		ACE inhibitory	1		
		Antiarrhythmic	1		
		Anticancer	1		
		Anticoagulant	1		
		Antitumor	1		
		Enzyme inhibitory	1		
		Reprod. system prot.	1		
		Sedative	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Crataegus nigra* Wald. Et Kit.** Hungarian hawthorn (crni glog)	Rosaceae	Antioxidant	1	In vivo mouse	1
		Anxiolytic	1		
***Crataegus pentagyna* Waldst. & Kit. ex Willd.** Small-flowered black hawthorn (petostubičasti glog)	Rosaceae	Antioxidant	2	In vitro assay systems	1
		UV protective	1	In vitro SPF test	1
Crataegus rhipidophylla Gand. Midland hawthorn (crveni glog)	Rosaceae	Antioxidant	3	In vitro assay systems	4
		Antioxidant	3	In vivo rat	4
		Cardioprotective	3	Human cell lines	3
		Antiapoptotic	1	Antimicrob. activ. ass.	1
		Antiatherogenic	1	Clinical trial	1
		Antimutagenic	1	In vitro rat	1
		Hypotensive	1	In vivo gerbil	1
		Mutagenic	1		
		ACE inhibitory	1		
		AChE inhibitory	1		
		Antiarrhythmic	1		
		Anticonvulsant	1		
		Antigenotoxic	1		
		Antiinflammatory	1		
		Antimicrobial	1		
		Genotoxic	1		
		Hepatoprotective	1		
***Elaeagnus rhamnoides* (L.) A. Nelson** Sea buckthorn (vučji trn) [synonym *Hippophae rhamnoides* L.]	Elaeagnaceae	Antioxidant	54	In vivo rat	34
		Cytoprotective	11	In vivo mouse	24
		Cardioprotective	10	In vitro assay systems	23
		Radioprotective	11	Human cell lines	22
		Hepatoprotective	9	Human volunteers	8
		Antiinflammatory	8	Mouse cell line	5
		Antiproliferative	6	Antimicrob. activ. ass.	4
		Wound healing	6	Inh. of bacterial grow.	4
		Antimicrobial	5	In vivo rabbit	3
		Antiplatelet aggreg.	5	Pharmacokinetic st.	3
		Hypoglycemic	5	Clinical trial	2

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
		Hypolipidemic	5	Double-blind study	2
		Neuroprotective	5	Toxicological study	2
		Antibacterial	4	In vivo chicken	1
		Antiulcerogenic	4	In vivo horse	1
		Antiaging	3	In vivo sheep	1
		Antiradical	3	Rat cell line	1
		Immunomodulatory	3		
		Proapoptotic	3		
		Anticancer	2		
		Antihypertensive	2		
		Antistress	2		
		Cytotoxic	2		
		Gastroprotective	2		
		Immunostimulatory	2		
		Retinoprotective	2		
		Skin protective	2		
		Antiapoptotic	1		
		Antiatherogenic	1		
		Anticoagulant	1		
		Antidepressant	1		
		Antifatigue	1		
		Antihypoxic	1		
		Antiobesity	1		
		Antithrombogenic	1		
		Antitumor	1		
		Anxiolytic	1		
		Enzyme inhibitory	1		
		Hypocholesterolemic	1		
		Hypotensive	1		
		Hypotriglyceridemic	1		
		Immunosuppressive	1		
		Lung protective	1		
		Spermicidal	1		
		UV protective	1		
***Fragaria moschata* (Duchesne) Duchesne** Musk strawberry (kitnjača)	Rosaceae	Antioxidant	1	In vitro assay systems	1
		Antiradical	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Fragaria vesca* L.** Wild strawberry (šumska jagoda)	Rosaceae	Antioxidant	9	In vitro assay systems	6
		Antibacterial	2	In vivo rat	3
		Antiinflammatory	2	Inh. of bacterial grow.	2
		Analgesic	1	Human cell lines	1
		Anticancer	1		
		Antiradical	1		
		Gastroprotective	1		
***Fragaria viridis* Weston** Green strawberry (jagoda pucavica)	Rosaceae	Antioxidant	1	In vitro assay systems	1
		Antiradical	1		
***Frangula alnus* Miller** Alder buckthorn (krušina)	Rhamnaceae	–		–	
***Frangula rupestris* Schur** Rock buckthorn (Stenjačka krušina)	Rhamnaceae	–		–	
***Juglans regia* L.** Common walnut (orah)	Juglandaceae	Antioxidant	22	Human cell lines	13
		Antimicrobial	6	In vitro assay systems	13
		Antiproliferative	6	Antimicrob. activ. ass.	5
		Antiinflammatory	4	In vivo rat	4
		Antiradical	3	Inh. of fungal growth	2
		Cytotoxic	3	Double-blind study	1
		Antifungal	2	In vivo mouse	1
		Nephroprotective	2	Mouse cell line	1
		Anticancer	1		
		Antihypertensive	1		
		Antihypertriglycerid	1		
		Antimutagenic	1		
		Antinociceptive	1		
		Antiplatelet aggreg.	1		
		Antitumor	1		
		Cardioprotective	1		
		Cytoprotective	1		
		Hepatoprotective	1		
		Lung protective	1		
		Neuroprotective	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Juniperus communis* L.** Common juniper (kleka)	Cupressaceae	Antioxidant	8	In vitro assay systems	8
		Antiinflammatory	4	Human cell lines	7
		Antimelanogenic	4	Antimicrob. activ. ass.	4
		Antimicrobial	4	In vivo rat	4
		Proapoptotic	3	In vivo zebrafish	2
		Anticancer	2	Inh. of fungal growth	2
		Antifungal	2	Mouse cell line	2
		Antiproliferative	2	In vivo nematode	1
		Antiaging	1	Inh. of bacterial grow.	1
		Antiarthritic	1	Toxicological study	1
		Antibacterial	1		
		Antifungal	1		
		Antinociceptive	1		
		Antitumor	1		
		Cytotoxic	1		
		Enzyme inhibitory	1		
		Genoprotective	1		
		Neuroprotective	1		
***Juniperus oxycedrus* L.** Cade juniper (crvena kleka)	Cupressaceae	Antioxidant	4	Human cell lines	4
		Antiinflammatory	3	In vitro assay systems	3
		Hypoglycemic	3	In vivo rat	3
		Antidiabetic	1	Antimicrob. activ. ass.	1
		Antimicrobial	1	Toxicological study	1
		Antinociceptive	1		
		Antiproliferative	1		
		Antiviral	1		
		Cytotoxic	1		
		Enzyme inhibitory	1		
***Lonicera alpigena* L.** Alpine honeysuckle (Alpsko pasje grožđe)	Caprifoliaceae	–		–	
***Lonicera caprifolium* L.** Italian woodbine (orlovi nokti)	Caprifoliaceae	–		–	

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Lonicera nigra* L.** Black-berried honeysuckle (crno pasje grožđe)	Caprifoliaceae	–		–	
***Lonicera xylosteum* L.** Fly honeysuckle (crveno pasje grožđe)	Caprifoliacea	–		–	
***Malus dasyphylla* Borkh.** Paradise apple (dlakavolisna jabuka)	Rosaceae	–		–	
***Malus florentina* (Zuccagni) C.K. Schneid.** *Florentine crabapple* (Firentinska jabuka)	Rosaceae	–		–	
***Malus sylvestris* (L.) Mill.** European crabapple (šumska jabuka)	Rosaceae	Antioxidant	2	In vitro assay systems	2
***Prunus amygdalus* Stokes** Almond (badem)	Rosaceae	Antioxidant	3	In vivo rat	2
		Antihyperlipidemic	1	Human cell lines	1
		Antiproliferative	1	In vitro assay systems	1
		Cytotoxic	1	Mouse cell line	1
		Gastroprotective	1		
		Hepatoprotective	1		
		Neuroprotective	1		
		Prebiotic	1		
***Prunus avium* (L.) L.** Wild cherry (trešnja vrapčara)	Rosaceae	Antioxidant	12	In vitro assay systems	9
		Antiradical	2	In vivo mouse	5
		Hepatoprotective	2	Human cell lines	2
		Radioprotective	2	Human volunteers	1
		Antifungal	1	In vivo mause	1
		Antitumor	1	Inh. of fungal growth	1
		Hypoglycemic	1	Mouse cell line	1
		Hypolipidemic	1		
		Immunostimulatory	1		
		Neuroprotective	1		
		Skin protective	1		
***Prunus cerasifera* Ehrh.** Cherry plum (džanarika)	Rosaceae	Antioxidant	3	In vitro assay systems	3
***Prunus fruticosa* Pall.** European dwarf cherry	Rosaceae	–		–	
***Prunus laurocerasus* L.** Cherry laurel (lovor višnja)	Rosaceae	Antioxidant	6	In vitro assay systems	6
		Antihyperglycemic	1	In vivo rat	1
		Antihyperlipidemic	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Prunus mahaleb* L.** Mahaleb cherry (magriva)	Rosaceae	Antioxidant	3	In vitro assay systems	2
		Antiinflammatory	2	Antimicrob. activ. ass.	1
		Antimutagenic	2	Human cell lines	1
		Antianaphylactic	1	In vivo guinea-pig	1
		Antimicrobial	1		
		Antiproliferative	1		
***Prunus padus* L.** European bird cherry (srezma)	Rosaceae	Antioxidant	3	In vitro assay systems	2
		Antibacterial	2	Inh. of bacterial grow.	2
		Antiradical	1		
***Prunus spinosa* L.** Blackthorn (crni trn)	Rosaceae	Antioxidant	14	In vitro assay systems	11
		Antibacterial	2	Antimicrob. activ. ass.	2
		Antimicrobial	2	Inh. of bacterial grow.	2
		Antiradical	2	Human cell lines	1
		Anticancer	1		
		Cytotoxic	1		
		Proapoptotic	1		
***Prunus tenella* Batsch** Dwarf Russian almond (stepski badem)	Rosaceae	–		–	
***Pyrus amygdaliformis* Vill.** Almond-leaved pear (slanopađa)	Rosaceae	–		–	
***Pyrus communis* L.** European pear (divlja kruška)	Rosaceae	Hypoglycemic	1	In vivo rat	1
		Hypolipidemic	1		
***Pyrus elaeagnifolia* Pall.** Oleaster-leafed pear (dafinolisna kruška)	Rosaceae	Antioxidant	1	In vitro assay systems	1
		Enzyme inhibitory	1		
***Pyrus nivalis* Jacq.** Snow pear (kasna kruška)	Rosaceae	–		–	
***Pyrus pyraster* (L.) Burgsd.** European wild pear (šumska kruška)	Rosaceae	–		–	
***Rhamnus alpina* L.** (žestika)	Rhamnaceae	–		–	
***Rhamnus cathartica* L.** Common buckthorn (pasdren)	Rhamnaceae	–		–	
***Rhamnus saxatilis* Jacq.** Rock buckthorn (kamenjarski pasjak)	Rhamnaceae	–		–	

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Ribes alpinum* L.** Mountain currant (planinska ribizla)	Grossulariaceae	–		–	
***Ribes multiflorum* Kit. ex Schult.** Manyflower currant (kitnjasta ribizla)	Grossulariaceae	Antioxidant	1	In vitro assay systems	1
***Ribes petraeum* Wulfen** Rock red currant (ribizla kamenjarka)	Grossulariaceae	–		–	
***Ribes uva-crispa* L.** Gooseberry (ogrozd)	Grossulariaceae	Antioxidant	4	In vitro assay systems	4
		Antifungal	1	Inh. of fungal growth	1
		Enzyme inhibitory	1		
***Rosa agrestis* Savi** Field briar (šipurak)	Rosaceae	–		–	
***Rosa andegavensis* Bastard**	Rosaceae	–		–	
***Rosa arvensis* Huds.** Field rose briar (poljska ruža)	Rosaceae	Antiinflammatory	1	Human cell lines	1
		Antioxidant	1		
***Rosa caesia* Sm.** Leder rose (kupinasta ruža)	Rosaceae	–		–	
***Rosa canina* L.** Dog rose (šipak)	Rosaceae	Antioxidant	32	In vitro assay systems	31
		Antiinflammatory	8	Human cell lines	12
		Antinociceptive	8	Double-blind study	7
		Antibacterial	5	In vivo rat	7
		Antiradical	5	In vivo mouse	3
		Antidiabetic	4	Clinical trial	2
		Antiproliferative	4	Controlled study	1
		Antihyperglycemic	3	Dog cell line	1
		Cytotoxic	3	Human volunteers	1
		Antiarthritic	2	In vitro antiviral test.	1
		Enzyme inhibitory	2	In vivo guinea-pig	1
		Nephroprotective	2	Inh. of bacterial grow.	1
		Neuroprotective	2	Mouse cell line	1
		Antiaging	1		
		Anticancer	1		
		Antihyperlipidemic	1		
		Antimelanogenic	1		
		Antimutagenic	1		
		Antiobesity	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
		Antiosteoporotic	1		
		Antitumor	1		
		Antiviral	1		
		Cardioprotective	1		
		Common cold protec.	1		
		Gastroprotective	1		
		Hepatoprotective	1		
		Hypotensive	1		
		Immunostimulatory	1		
		Skin protective	1		
		Uroprotective	1		
***Rosa dumetorum* Thvill.** (živična ruža)	Rosaceae	Antiinflammatory	1	Human cell lines	1
		Cytotoxic	1		
***Rosa foetida* Herrm.** Austrian briar	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rosa gallica* L.** Gallic rose (Galska ruža)	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rosa glauca* Pourr.** Redleaf rose (modrolisna ruža)	Rosaceae	–		–	
***Rosa glutinosa* Sibth. & Sm.** (lepljiva ruža)	Rosaceae	–		–	
***Rosa micrantha* Borrer ex Sm.** Smallflower sweetbrier	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rosa nitidula* Besser**	Rosaceae	–		–	
***Rosa obtusifolia* Desv.**	Rosaceae	–		–	
***Rosa pendulina* L.** Mountain rose (planinska ruža)	Rosaceae	–		–	
***Rosa rubiginosa* L.** Sweetbriar rose (vinska ruža)	Rosaceae	Antioxidant	3	In vitro assay systems	
		Antiobesity	1	In vivo mouse	1
		Hepatoprotective	1		
***Rosa spinosissima* L.** Scotch rose	Rosaceae	–		–	
***Rosa tomentosa* Sm.** Whitewoolly rose (maljava ruža)	Rosaceae	–		–	
***Rosa villosa* L.** Apple rose (jabukolika ruža)	Rosaceae	Antioxidant	1	In vitro assay systems	1
		Antiproliferative	1	Human cell lines	1
***Rubus caesius* L.** European dewberry (kupina-ostruga)	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rubus candicans* Weihe ex Rchb.** (stublasta kupina)	Rosaceae	–		–	

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Rubus canescens* Dc**. Woolly blackberry (sivkasta kupina)	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rubus fruticosus* L**. Blackberry (kupinjača)	Rosaceae	Antioxidant	5	In vitro assay systems	4
		Enzyme inhibitory	2	Antimicrob. activ. ass.	1
		Antiinflammatory	1	Human volunteers	1
		Antimicrobial	1	In vivo rabbit	1
		Antispasmodic	1	In vivo rat	1
		Antiparasitic	1		
		Gastroprotective	1		
***Rubus glandulosus* Bellardi** (žlezdasta kupina)	Rosaceae	Anxiolytic	1	In vivo mouse	1
***Rubus hirtus* Waldst. & Kit**. (dlakava kupina)	Rosaceae	Antioxidant	1	In vitro assay systems	1
***Rubus idaeus* L**. European raspberry (malina)	Rosaceae	Antioxidant	12	In vitro assay systems	10
		Antimetastatic	5	Human cell lines	7
		Antibacterial	2	Antimicrob. activ. ass.	1
		Antiproliferative	2	In vivo mouse	1
		Antiradical	2	In vivo mouse + h. c. lin	1
		AGE inhibition	1	In vivo rat	1
		Antifungal	1	Inh. of bacterial grow.	1
		Antiinflammatory	1	Inh. of fungal growth	1
		Antimicrobial	1		
		Antitumor	1		
		Cytotoxic	1		
		Diuretic	1		
		Hypolipidemic	1		
		Neuroprotective	1		
		Proapoptotic	1		
		UV protective	1		
***Rubus plicatus* Weihe & Nees** Plaited-leaved bramble (kupina)	Rosaceae	–		–	
***Rubus saxatilis* L**. Stone bramble (kupina kamenjarka)	Rosaceae				

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Rubus ulmifolius* Schott** Elmleaf blackberry (kupinjača)	Rosaceae	Antioxidant	7	Antimicrob. activ. ass.	1
		Antibacterial	1	Brine shrimp leth. b.	1
		Antihyperlipidemic	1	In vitro antiviral test	1
		Antiinflammatory	1	In vivo rat	1
		Antimicrobial	1	Inh. of bacterial grow.	1
		Antiparasitic	1		
		Antipyretic	1		
		Antiradical	1		
		Antiviral	1		
		Hypoglycemic	1		
***Sambucus ebulus* L.** Danewort (burjan)	Adoxaceae	Antioxidant	9	In vitro assay systems	8
		Antiinflammatory	2	Human cell lines	2
		Antiproliferative	2	Toxicological study	2
		Enzyme inhibitory	2	Antimicrob. activ. ass.	1
		Anticolitis	1	Double-blind study	1
		Antidepressant	1	Human volunteers	1
		Antidiabetic	1	In vitro antiviral test	1
		Antiemetic	1	In vitro sheep	1
		Antimicrobial	1	In vivo chicken	1
		Antiparasitic	1	In vivo mouse	1
		Antiradical	1	In vivo rat	1
		Antiviral	1	Inh. of the parasite gr.	1
		Cytotoxic	1	Mouse cell line	1
		Gastroprotective	1		
		Hypolipidemic	1		
		Neuroprotective	1		
		Scolicidal	1		
		Wound-healing	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Sambucus nigra* L.** European elder (crna zova)	Adoxaceae	Antioxidant	14	In vitro assay systems	11
		Antiinflammatory	5	In vivo rat	6
		Hypoglycemic	4	Antimicrob. activ. ass	2
		Antimicrobial	2	Human cell lines	2
		Antiviral	2	In vitro antiviral test.	2
		Hypolipidemic	2	In vivo mouse	2
		AChE inhibitory	1	Human + murine c. l.	1
		Antibacterial	1	Inh. of bacterial grow.	1
		Anticonvulsant	1	Inh. of the parasite gr.	1
		Antidepressant	1	Mouse cell line	1
		Antiepileptic	1	Pharmacokinetic study	1
		Antihypertensive	1		
		Antimutagenic	1		
		Antiosteoporotic	1		
		Antiparasitic	1		
		Antiproliferative	1		
		Immunomodulatory	1		
		Proapoptotic	1		
***Sambucus racemosa* L.** (Red elderberry) crvena zova	Adoxaceae	–		–	
***Sorbus aria* (L.) Crantz** Common whitebeam (mukinja)	Rosaceae	Antioxidant	3	In vitro assay systems	3
***Sorbus aucuparia* L.** Rowan (jarebika)	Rosaceae	Antioxidant	3	In vitro assay systems	3
		Antimetastatic	1	In vivo mouse	1
		Antitumor	1	Mouse cell line	1
		Hypoglycemic	1		
		Hypolipidemic	1		
***Sorbus austriaca* (Beck) Hedl.** Austrian whitebeam (Austrijska mukinja)	Rosaceae	–		–	
***Sorbus chamaemespilus* (L.) Crantz** Dwarf whitebeam (mukijica)	Rosaceae	–		–	
***Sorbus domestica* L.** Service tree (oskoruša)	Rosaceae	Antioxidant	3	In vitro assay systems	4
		Enzyme inhibitory	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
***Sorbus graeca* (Lodd. ex Spach) Kotschy** Greek whitebeam (grčka mukinja)	Rosaceae	–		–	
***Sorbus mougeotii* Soy.-Will. & Godr.** Mougeot's whitebeam	Rosaceae	–		–	
***Sorbus torminalis* (L.) Crantz** Wild service tree (brekinja)	Rosaceae	Antioxidant	3	In vitro assay systems	3
		AChE inhibitory	1		
***Sorbus umbellata* (Desf.) Fritsch** Mountain ash (štitasta mukinja)	Rosaceae	Antioxidant	2	In vitro assay systems	2
		AChE inhibitory	1		
***Taxus baccata* L.** Yew (tisa)	Taxaceae	Anticancer	1	Human cell lines	1
		Antioxidant	1		
***Vaccinium myrtillus* L.** European blueberry (borovnica)	Ericaceae	Antioxidant	22	In vitro assay systems	10
		Antiinflammatory	5	In vitro assay systems	10
		Cardioprotective	5	In vivo mouse	9
		Hypoglycemic	4	In vivo rat	9
		Cytoprotective	3	Human cell lines	8
		Hepatoprotective	3	Human volunteers	3
		Nephroprotective	3	In vitro rat	2
		Retinoprotective	3	In vivo mouse + h. c. lin.	2
		Antidiabetic	2	In vivo rabbit	2
		Antiproliferative	2	Pharmacokinetic study	2
		Antiradical	2	Antimicrob. activ. ass.	1
		Neuroprotective	2	Double-blind study	1
		Antiatherogenic	1	In vivo hamster	1
		Antibacterial	1	Inh. of bacterial grow.	1
		Antidepressant	1	Inh. of fungal growth	1
		Antifungal	1		
		Antihyperglycemic	1		
		Antihyperlipidemic	1		
		Antimicrobial	1		
		Antiobesity	1		
		Antipruritic	1		
		Antitumor	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
		Enzyme inhibitory	1		
		Gastroprotective	1		
		Hypolipidemic	1		
		Ophthalmic protective	1		
		Proapoptotic	1		
		Toxicity protective	1		
		UV protective	1		
***Vaccinium uliginosum* L.** Bog bilberry (plava borovnica)	Ericaceae	Antioxidant	7	In vitro assay systems	7
		Retinoprotective	3	Human cell lines	2
		Skin protective	2	In vivo mouse + h. c. lin.	2
		Anticancer	1	In vivo mouse	2
		Antiinflammatory	1	Double-blind study	1
		Antiproliferative	1	Human + murine c. l.	1
		Antiradical	1	In vivo rabbit	1
		Antispasmodic	1		
		Immunoregulatory	1		
		Ophthalmic protective	1		
***Vaccinium vitis-idaea* L.** Lingonberry (brusnica)	Ericaceae	antioxidant	9	In vitro assay systems	9
		Cardioprotective	6	Human cell lines	7
		Antidiabetic	3	Antimicrob. activ. ass.	3
		Antimicrobial	3	In vitro antiviral test.	2
		Antiviral	2	In vivo mouse	2
		Hypoglycemic	2	In vivo rat	1
		AChE inhibitory	1		
		AGE inhibition	1		
		Antiproliferative	1		
		Hepatoprotective	1		
		Hypolipidemic	1		
		Proapoptotic	1		
***Viburnum lantana* L.** Wayfaring tree (crna udika)	Adoxaceae	Antioxidant	2	In vitro assay systems	4
		AChE inhibitory	1	Antimicrob. activ. ass.	1
		Antibacterial	1		

(*Continued*)

TABLE 10.1 (Continued)

Species common name (in Serbian)	Family	Biological and pharmacological activity		Study protocol	
		Antimicrobial	1		
		Antitumor	1		
***Viburnum opulus* L.** Guelder rose (crvena udika)	Adoxaceae	Antioxidant	11	In vitro assay systems	9
		Antimicrobial	4	In vivo rat	7
		Antibacterial	2	Antimicrob. activ. ass.	4
		Anticancer	2	In vivo mouse	2
		Antiradical	2	Inh. of bacterial grow	2
		Nephroprotective	2	Human cell lines	1
		AChE inhibitory	1		
		Antiendometriotic	1		
		Antiurolithiatic	1		
		Diuretic	1		
		Gastroprotective	1		
		Immunostimulatory	1		
		Lung protective	1		
		Reprod. system prot.	1		
Vitis vinifera* subsp. *sylvestris* (C.C. *Gmel.) Hegi Wild grape (šumska loza)	Vitaceae	Antioxidant	1	In vitro assay systems	1
		Antiradical	1		

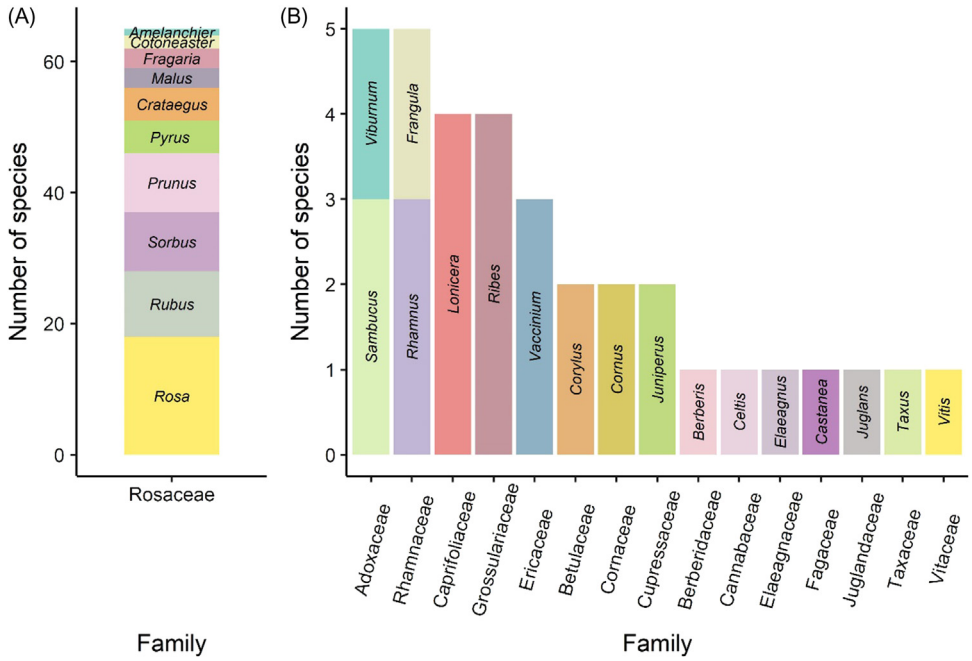

FIGURE 10.2 The number of wild-fruit species in Serbia belonging to family Rosaceae (A) and other plant families (B).

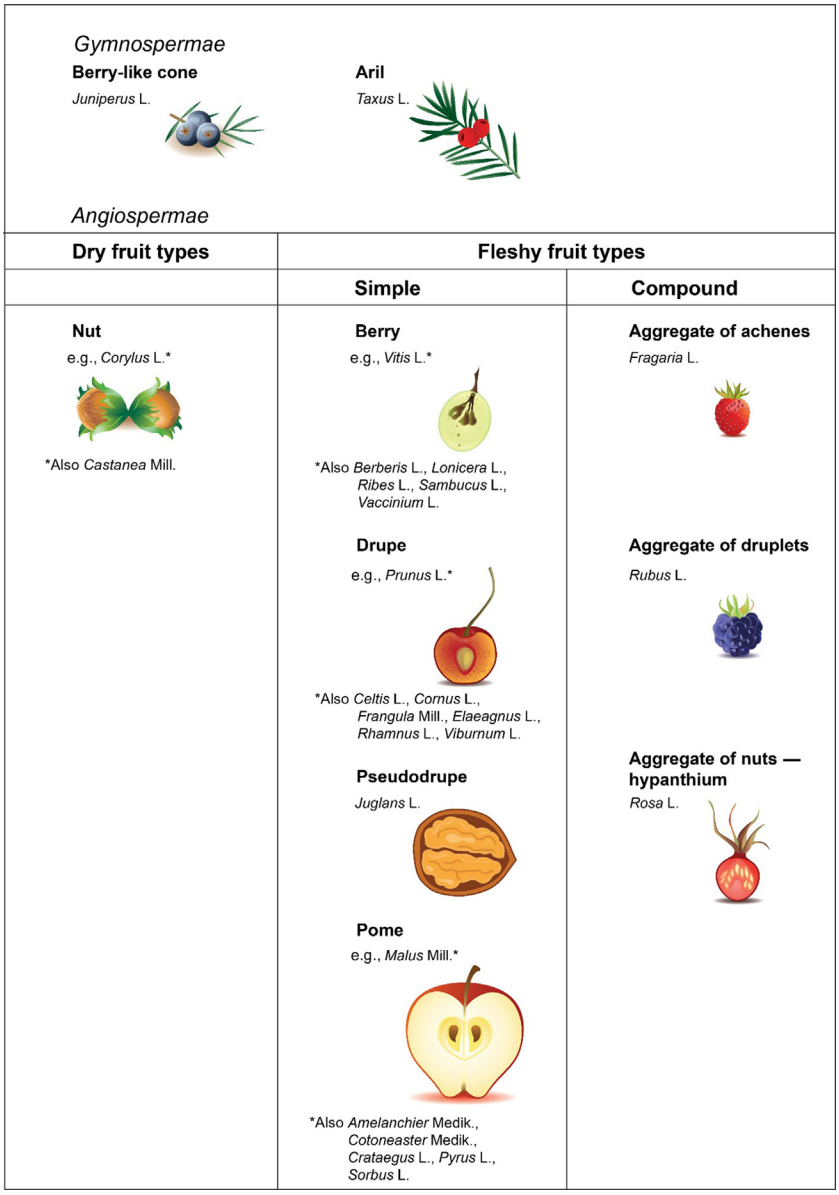

FIGURE 10.3 Different fruit types of investigated species.

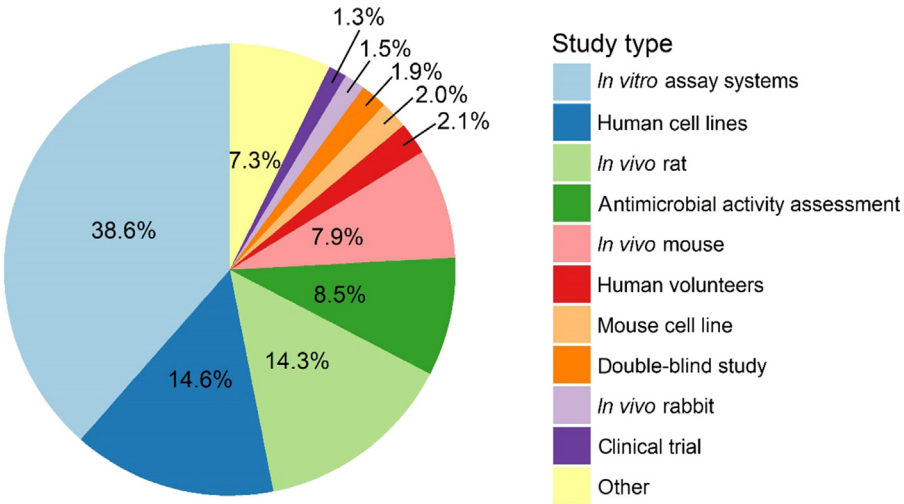

FIGURE 10.4 Different types of studies for determining the biological activity of wild fruits.

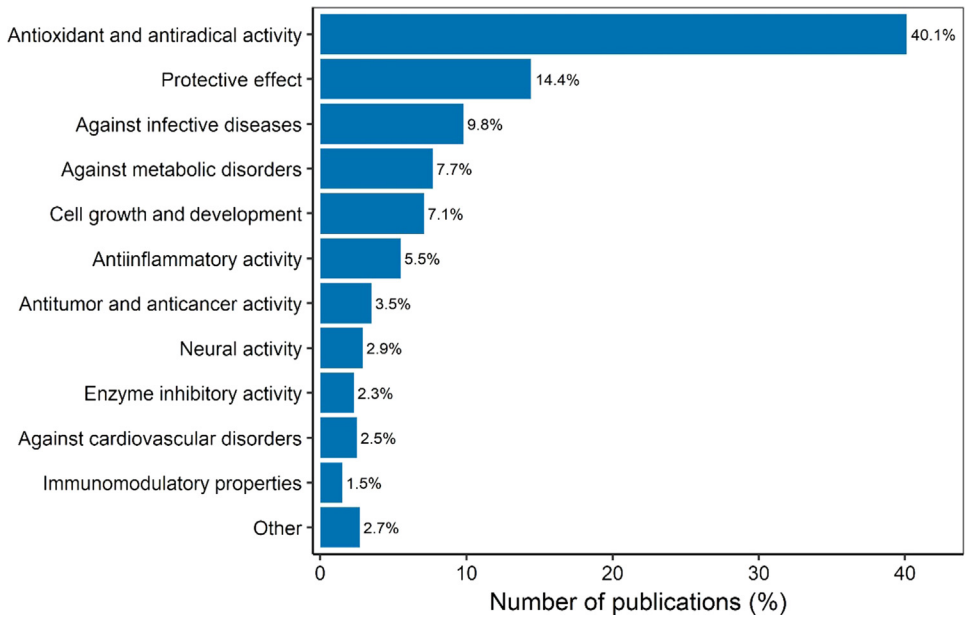

FIGURE 10.5 Main groups of biomedical activities of wild fruits.

signaling (Bókkon, 2012). Their extensive production is recognized as a central factor in many chronic diseases and numerous studies of various natural products have demonstrated their ability to suppress free radical activity. In determining the effectiveness of tested compounds against free radical activity, antioxidant and antiradical activities should be differentiated. Antiradical activity is the ability of compounds to react with free radicals (a single reaction), whereas antioxidant activity is characterized by the ability to inhibit the process of oxidation (usually a set of different reactions) (Burlakova, Alesenko, Molochkina, Palmina, & Khrapova, 1975). For testing antiradical activity, DPPH and galvinoxyl tests are usually used, and further studies on the antioxidant activity of these compounds have shown that their antiradical activities do not necessarily correspond to antioxidant activity; for determination of total antioxidative capacity, ABTS-based test systems and hydroxyl radical scavenging activity tests are usually used (Tirzitis & Bartosz, 2010). Considering that most studies are carried out in test systems, the actual in vivo antioxidant activity should be confirmed in studies on the real product; also, the interpretation of results of the antiradical and antioxidant potential of tested compounds should be especially carefully interpreted if clinical samples (blood plasma) are subjected to analysis (Tirzitis & Bartosz, 2010). Antiradical and antioxidant activities of listed plants were examined in 446 studies. Out of the total number of tested species, 33 wild fruits showed antioxidant activity; the highest number of records confirmed the antioxidant activity of *Elaeagnus rhamnoides* (28), *Cornus mas* (10), *Juglans regia* (9), *Vaccinium myrtillus* (9), *Rosa canina* (8), and *Berberis vulgaris* (5). Of these 33 species, 15 showed both antiradical and antioxidant activities: in addition to the already mentioned species are *Corylus avellana*, *Fragaria vesca*, *F. moschata*, *F. viridis*, *Prunus avium*, *P. padus*, *P. spinosa*, *Rubus idaeus*, *Vaccinium uliginosum*, and *Viburnum opulus*. A smaller number of species were tested in in vitro systems, while 24 fruits confirmed antiradical/antioxidant activity in human cell lines (*B. vulgaris*, *Castanea sativa*, *C. mas*, *Crataegus monogyna*, *C. riphidiphylla*, *Juglans regia*, *Juniperus communis*, *Prunus amygdalus*, *P. mahaleb*, *P. spinosa*, *Rosa dumeitorum*, *R. villosa*, *R. idaeus*, *Sambucus nigra*, *V. uliginosum*, *V. vitis-idaea*, *V. opulus*, and *Taxus baccata*) or both in human cell lines and in human volunteers (*C. avellana*, *E. rhamnoides*, *P. avium*, *R. canina*, *Sambucus ebulus*, and *V. myrtillus*).

10.6.2 Protective effect

The protective effects resulting from the intake of natural products based on wild fruits' extracts was shown in numerous studies. These effects in various extracts of natural origin are mostly attributed to their antioxidant, antimutagenic, antigenotoxic, apoptotic, and cytotoxic properties (Reddy, Odhav, & Bhoola, 2003). Studies (160) analyzed in this review showed the protective potential of wild fruits on different organ systems, organs, and tissues. Regarding model systems, the highest number of species was tested in in vivo animal models, with a few species confirming protective effects in human cell lines (*E. rhamnoides*, *J. regia*, *V. myrtillus*, *V. vitis-idaea*) and human volunteers (*E. rhamnoides*, *R. canina*), and only three species were tested in in vitro assay systems. A cardioprotective effect was exhibited by the fruits of *C. mas*, *C. monogyna*, *E. rhamnoides*, *R. canina*, *V. myrtillus*, cytoprotective by *C. sativa*, *C. mas*,

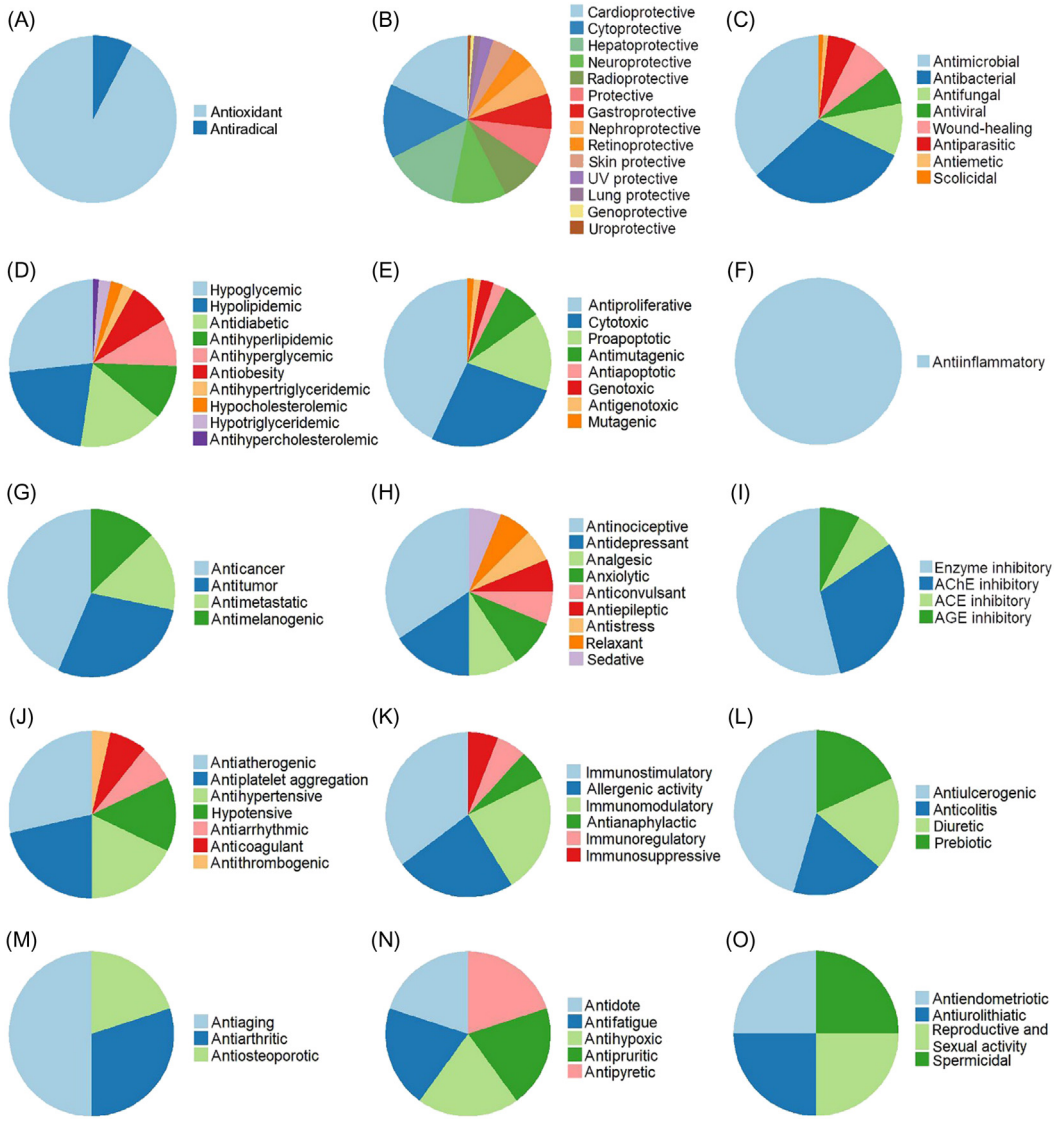

FIGURE 10.6 Specific biomedical activities of wild fruits within the major groups (A) antioxidant and antiradical activity, (B) protective effect, (C) against infective diseases, (D) metabolic disorders, (E) activity in cell growth and development, (F) antiinflammatory activity, (G) antitumor and anticancer activity, (H) neural activity, (I) enzyme inhibitory activity, (J) against cardiovascular disorders, (K) immunomodulatory activity, (L) against gastrointestinal disorders, (M) against age-related disorders, (N) against general health disorders, (O) against reproductive system disorders.

C. monogyna, *E. rhamnoides*, *J. regia*, *V. myrtillus*, *V. vitis-idaea*, hepatoprotective by *Crataegus rhipidophylla*, *E. rhamnoides*, *J. regia*, *P. amygdalus*, *P. avium*, *R. canina*, *R. rubiginosa*, and *V. myrtillus*, neuroprotective by *E. rhamnoides*, *J. communis*, *P. avium*, *R. canina*, *R. idaeus*, *V. myrtillus*; gastroprotective by *E. rhamnoides*, *P. amygdalus*, *R. canina, V. myrtillus*, *V. opulus*, *E. rhamnoides*, and *J. regia*; and *V. opulus* also showed a lung-protective effect. Skin protective effect was shown by fruits of *R. canina*, *P. avium*, *V. uliginosum*, *C. mas*, and *E. rhamnoides*; UV protective effect by fruits of *Crataegus pentagyna*, *E. rhamnoides*, *R. idaeus*, and *V. myrtillus*; and radioprotective effect by fruits of *E. rhamnoides* and *P. avium*. Fruits of *V. myrtillus*, *V. uliginosum*, and *V. opulus* showed nephroprotective, retinoprotective, and uroprotective effects. The highest number of protective properties was attributed to the fruits of *E. rhamnoides* (9), *V. myrtillus* (8), *R. canina* (5), and *V. opulus* (5).

10.6.3 Against disease-causing organisms

A wide array of causative agents of infectious and noninfectious diseases was used in biotesting the suppressive activities of wild fruits. Antimicrobial testing was the most common in this group of studies. Twenty plant species tested for

this activity showed an inhibitory effect on both bacterial and fungal growth; among them, the most frequently used were *E. rhamnoides*, *J. regia*, *J. communis*, and *V. opulus*. Antibacterial activity (studied in 34 articles) was attributed to the fruit extracts of 15 species that showed inhibitory effect on bacterial growth when applied to cultures of various pathogenic bacterial lines. The majority of articles revealed the antibacterial activity of *E. rhamnoides*, *R. canina*, *V. opulus*, and *Prunus spinosa*. The antifungal activity of wild fruits was mostly tested against *Aspergillus* and *Candida* strains, and inhibition of fungal growth was achieved by the application of *J. regia*, *J. communis*, *P. avium*, *Ribes uva-crispa*, *R. idaeus*, and *V. myrtillus* fruits. Antiviral activity (against herpes, influenza, bronchitis viruses) was shown by the fruits *of R. canina*, *Rubus ulmifolius*, *S. ebulus*, *S. nigra*, and *Vaccinium vitis-idaea*. *S. ebulus* and *S. nigra* showed antiparasitic and antiemetic activity. The wound-healing activity of fruit extracts is mostly based on their antimicrobial, hemostatic, antiinflammation, proliferative, and reepitalization properties (Janis and Harrison, 2014). In the reviewed articles, wound-healing activity was associated with the ability to heal scars and wounds after topical application in animal models, and this effect was shown by *E. rhamnoides* fruits.

10.6.4 Against metabolic disorders

Imbalances in various metabolic pathways are often a consequence of inherited metabolic diseases, but may also be caused by endocrine diseases, bad diet, a sedentary lifestyle, stress, aging, etc., which also affect cellular metabolism. Metabolic disorders can be manifested as obesity, type 2 diabetes, metabolic syndrome, nonalcoholic fatty liver disease and hyperlipidemia, and others (Heindel et al., 2017). The positive effects of wild fruits against disorders in carbohydrate metabolism were revealed in several studies: an antihyperglycemic effect was shown by the fruits of *C. mas*, *Prunus laurocerasus*, *R. canina*, and *V. myrtillus* in in vivo animal models; a hypoglycemic effect was shown by the fruits of *B. vulgaris*, *Juniperus oxycedrus*, *Pyrus communis*, *P. avium*, *R. ulmifolius*, *S. nigra*, *Sorbus aucuparia*, and *V. vitis-idaea* in in vivo animal models, and *E. rhamnoides* and *V. myrtillus* in human volunteers. An antiobesity effect of the fruits of *C. mas* and *E. rhamnoides* was revealed in in vivo animal studies and an antidiabetic effect of the fruits of *B. vulgaris*, *R. canina*, and *V. vitis-idaea* in human cell lines. Disorders in lipid metabolism were studied by testing the effects of a few wild fruits. *C. mas* fruits showed hypolipidemic, hypotriglyceridemic, hypocholesterolemic, antihypertriglyceridemic, and antihyperlipidemic effects in animal in vivo models, human cell lines, and in human volunteers. Some of these activities were shown by the fruits of *E. rhamnoides*, *J. regia*, *R. idaeus*, *S. ebulus*, and *P. amygdalus*.

10.6.5 Activity in cell growth and development

The life cycle of a cell relies on constant DNA reading for the synthesis of a particular set of proteins. These proteins perform specific functions, undergo degradation, and are replaced with new proteins to fulfill their static and dynamic functions (Lodish et al., 2000). The main phases of cellular dynamics are cell formation, growth, differentiation, and division or death, and each of these points is strictly encoded and regulated by multiple mechanisms. Disorders in cellular dynamic pathways may lead to serious health problems, and numerous studies have aimed to determine the effects of different causes both on the disturbance and the establishment of normal cell dynamics. All studies presented herein were carried out on human cell lines, and antiproliferative activity was shown by 15 fruits: *C. sativa*, *C. mas*, *E. rhamnoides*, *J. regia*, *J. communis*, *J. oxycedrus*, *P. amygdalus*, *P. mahaleb*, *R. canina*, *R. villosa*, *R. idaeus*, *S. ebulus*, *V. myrtillus*, *V. uliginosum*, and *V. vitis-idaea*. Antimetastatic activity was shown by *R. idaeus* fruits, and antimutagenic activity by the fruits of *R. canina* and *S. nigra*.

10.6.6 Antiinflammatory activity

Inflammation as a part of the immune response to injury or infection is usually acute and lasts throughout injury healing. However, it is also firmly related to various physiopathological processes, with chronic and systemic effects/manifestations (Mathur & Pedersen, 2008). Although short-term inflammation is successfully suppressed by nonsteroidal antiinflammatory drugs, chronic inflammation (such as asthma, allergy, and arthritis) is usually treated with corticosteroids, which may exhibit some side effects. Considering their natural origin and proven antiinflammatory properties, some wild fruits are among potential sources for antiinflammatory products. Activities against induced or preexisting inflammation were carried out on in vivo animal models, human cell lines, and in one clinical trial. Antiinflammatory activity was confirmed for the fruits of 19 species: *B. vulgaris*, *C. mas*, *C. avellana*, *C. monogyna*, *C. rhipidophylla*,

F. vesca, *E. rhamnoides*, *J. regia*, *J. communis*, *J. oxycedrus*, *Rosa arvensis*, *R. canina*, *Rubus fruticosus*, *R. idaeus*, *R. ulmifolius*, *S. ebulus*, *S. nigra*, *V. myrtillus*, and *V. uliginosum*; an antiinflammatory effect of *C. mas* supplementation was revealed in one clinical trial conducted with dyslipidemic children and adolescents.

10.6.7 Antitumor and anticancer activities

The large number and diversity of natural compounds and their multiple biological activities provide a promising basis for the search for modern cytotoxic agents and anticancer drugs. Some compounds of natural origin were modified to enhance their therapeutic potential, pharmacological activity, and to lower their side effects (Cragg, Grothaus, & Newman, 2009; Gordaliza, 2007). The antitumor and anticancer activities of 15 tested fruit species were investigated in in vivo animal models, human cell lines, and in one clinical trial. The fruits of *C. mas*, *C. monogyna*, *E. rhamnoides*, *J. regia*, *J. communis*, *P. avium*, *R. canina*, *S. aucuparia*, *V. myrtillus*, and *Viburnum lantana* showed antitumor activity. *B. vulgaris* was tested for anticancer activity in a clinical trial with women with benign breast disease, and the following species showed anticancer activity in animal models and/or human cell lines: *C. monogyna*, *F. vesca*, *E. rhamnoides*, *R. canina*, *V. uliginosum*, *V. opulus*, and *T. baccata*.

10.6.8 Neural and psychotic activities

The powerful antioxidant activities of wild fruit products, along with their antiacetylcholinesterase activity, protective effects against neurotoxicity, and potency against hyperphosphorylation and beta amyloid accumulation, support the need for further research into their application as neuroprotective and antineurodegenerative agents (Ebrahimi & Schluesener, 2012). The largest number of studies in this group, including two clinical trials, revealed the antinociceptive activity of *R. canina* fruits. Anxiolytic activity was shown by the fruits of *Crataegus nigra*, *E. rhamnoides*, and *Rubus glandulous*; antiepileptic activity by *C. mas* and *S. nigra*; antidepressant activity by *S. ebulus*, *S. nigra*, and *E. rhamnoides*; sedative activity by *C. monogyna*; and anticonvulsant activity by *C. rhipidophylla*.

10.6.9 Enzyme inhibitory activity

Enzyme inhibitory activity is the ability of a molecule to bind to an enzyme and suppress or inhibit its activity. The principle of enzyme inhibition is the mode of action of many conventional drugs, characterized by high specificity, potency, and low toxicity. New drugs are also developing through the testing of numerous chemical compounds against targeted enzymes responsible for different physiopathological conditions (Scapin, 2006). Several enzymes were used in studies of the inhibitory activity of wild fruits: angiotensin-converting enzyme (ACE), acetylcholinesterase (AChE), lipase and lipoxygenase, polyphenol oxidase, antixanthine oxidase, antiurease, α-glucosidase, hyaluronidase, and aldose reductase. Most of these studies were carried out in in vitro assay systems, and only a few in human cell lines. Enzyme inhibitory activity was shown by the fruits of 16 investigated species: *C. monogyna*, *C. rhipidophylla*, *E. rhamnoides*, *J. communis*, *J. oxycedrus*, *Pyrus elaeagnifolia*, *R. canina*, *R. fruticosus*, *R. idaeus*, *S. ebulus*, *Sorbus domestica*, *S. umbellata*, *V. myrtillus*, *V. vitis-idaea*, *V. lantana*, and *V. opulus*.

10.6.10 Against cardiovascular disorders

The consumption of fruits is inversely correlated with risk of cardiovascular diseases, which are a major global health concern. Wild fruits show protective and therapeutic functions against CVD (preventing or restoring postinjury morphology and functions of heart and vessels) through multiple mechanisms: improvement of vascular endothelial function, balancing of blood pressure and lipid metabolism, and as antioxidative, antiinflammatory, antithrombotic, and antiplatelet agents (Zhao et al., 2017). Besides the species mentioned above for their cardioprotective activity, 12 wild fruits showed activity against various disorders related to the cardiovascular system (Table 10.1). The highest number of beneficial activities was determined for *E. rhamnoides* in in vivo animal studies, human cell lines, and in human volunteers (antithrombogenic, anticoagulant, vasculoprotective, antiplatelet aggregation, hypotensive, and antiatherogenic). Antiarrhythmic activity was shown by the fruits of *C. monogyna*; antihypertensive activity by *B. vulgaris*, *J. regia*, and *S. nigra*; and hypotensive activity by *C. mas*.

10.6.11 Immunomodulatory activity

Some wild-fruit products can alter the immune response by either enhancing or suppressing it. Both immunoenhancing/immunostimulatory (for preventing and fighting infection) and immunosuppressive (for preventing the rejection of transplanted organs/tissues and for treating various autoimmune diseases or nonautoimmune diseases) effects include several activities, i.e., preservation of plasma membrane integrity, macrophage production, immunoglobulin synthesis, effects on signal transduction pathways, and others, all of them being particularly sensitive to oxidative stress (Grigore, 2017). The ability of immunomodulation was revealed for the fruits of *E. rhamnoides*, *P. avium*, *R. canina*, *S. nigra*, and *V. opulus* in human cell lines and in in vivo animal models. *C. avellana* fruits showed allergenic activity both in human cell lines and in human volunteers.

10.6.12 Against gastrointestinal disorders

Many gastrointestinal (GI) disorders and digestive problems can be prevented or treated by an appropriate diet. Wild edible fruits, possessing both nutritive and health-enhancing qualities, are considered functional foods. Current ethnobotanical literature suggests that 10%−50% of GI disorders are treated using functional foods (Valussi, 2012). A beneficial effect on the GI system (including antiinflammatory, antioxidative, antiulcerogenic, and prebiotic activities) of five wild fruits was revealed in in vivo animal studies: *E. rhamnoides*, *P. amygdalus*, *R. canina*, *V. myrtillus*, and *V. opulus*.

10.6.13 Against age-related disorders

There are numerous studies of age-related disorders aimed at the prevention and improvement of degenerative processes for healthy aging. The most researched topics are the accumulation of ROS-induced damage and the development of age-related metabolic diseases (Waltenberger et al., 2018). Studies investigating the activities of wild fruits in age-related disorders have shown a promising effect of *J. communis* fruits in in vivo animal models, and *E. rhamnoides* and *R. canina* in human volunteers.

10.6.14 Against general health disorders

General altered health conditions are manifestations of various disorders in physical and/or mental functioning, and agents that successfully cure the conditions usually have a beneficial effect on several functions that have been disturbed. The fruits of *E. rhamnoides* have shown beneficial effect against fatigue and hypoxia, fruits of *R. ulmifolius* against asymptomatic temperature, and fruits of *V. myrtillus* against skin irritation.

10.6.15 Against reproductive system disorders

Reproductive system protection and hormonal regulation was shown in in vivo animal studies by the fruits of *C. monogyna* and *V. opulus*, and the fruits of *E. rhamnoides* showed spermicidal activity in human volunteers.

10.7 Species with a pronounced biomedical potential

Most of the listed species were subjected to phytochemical investigation and testing on biological activities (60), but there are a number of species that have not been studied yet (38). According to the articles reviewed herein, the most examined species was *E. rhamnoides*, reported in 140 studies, with 43 biological activities confirmed in in vitro and in vivo assay systems, in vivo animal models, human cell lines, in human volunteers, and in clinical trials. The fruits of *R. canina* were studied in 70 articles, and 13 different biological activities were revealed; the fruits of *C. mas* (57 studies) showed 21 biological activities; the fruits of *V. myrtillus* (52 studies), 10 biological activities; fruits of *J. regia* (40 studies), 15 biological activities; fruits of *J. communis* (32 studies), 15 biological activities; fruits of *C. monogyna* and *S. nigra* (each species 30 studies), both displaying 14 activities; the fruits of *B. vulgaris* and *R. idaeus* (each species 24 studies), 13 and 12 activities, respectively. Wild-fruit species that were included in clinical trials (reported in 10 articles) were *B. vulgaris*, *C. mas*, *C. monogyna*, *C. rhipidophylla*, *E. rhamnoides*, and *R. canina*; those tested in human volunteers (18 articles) were *C. avellana*, *E. rhamnoides*, *P. avium*, *R. canina*, *R. fruticosus*, *S. ebulus*, and *V. myrtillus*. The biological activities and biomedical potential of the most studied plants have been previously reviewed (Alasalvar & Bolling, 2015; Chrubasik, Duke, & Chrubasik, 2006; De Vasconcelos, Bennett, Rosa, & Ferreira-Cardoso, 2010; Dinda et al., 2016; Esfahlan, Jamei, & Esfahlan, 2010; Hayes, Angove, Tucci, & Dennis, 2016;

Imanshahidi & Hosseinzadeh, 2008; Imenshahidi & Hosseinzadeh, 2016; Nabavi et al., 2015; Orhan, 2018; Patel, 2017; Shokrzadeh & Saravi, 2010; Suryakumar & Gupta, 2011; Szajdek & Borowska, 2008; Zia-Ul-Haq, Riaz, De Feo, Jaafar, & Moga, 2014).

The great biodiversity of wild fruits in Serbia (99 species) and the available evidence of their numerous biological activities indicate that this plant group should be further investigated in phytochemical and biomedical studies. Considering that the fruits of 60 species exhibited biological activities of biomedical importance, and that there are still no data for many of the listed species, the necessity to protect wild fruits in natural habitats, their further investigation, and larger incorporation in human studies becomes evident.

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Chapter 11

Botanicals from the Himalayas with anticancer potential: an emphasis on the Kashmir Himalayas

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11.1 Introduction

Medicinal plants have been used as the primary source in traditional medicine for the treatment of various diseases for thousands of years in Africa, China, Central Asia, Greece, India, Indonesia, Pakistan, Malaysia, Turkey, and the Americas. They have been evaluated for their natural antiseptic, antioxidant, and anticancer activities. Even today a large percentage of humankind around the globe depends on medicinal plants for their primary health care (Greenwell & Rahman, 2015; Ozturk and Hakeem, 2018, 2019a, 2019b). A number of modern drugs have been developed and plant secondary metabolites have been proven to be a great reservoir for new compounds of medicinal value (Kainsa & Kumar, 2012). The World Health Organization (WHO) report reports that the annual demand for medicinal plants at present is around US$14 billion, and it is expected to reach a level of US$5 trillion by 2050 (Dar et al., 2017; Wakdikar, 2004). According to Barata, Rocha, Lopes, and Carvalho (2016) WHO has listed 20,000 plant taxa up until now and efforts are being made to identify medicinal plants at the global level, although indiscriminate harvesting of these plants is seriously damaging the medicinal plant diversity.

Cancer is amongst the foremost causes of deaths all over the world and still remains as one of the most threatening fatal diseases. Many of the novel synthetic chemotherapeutic agents used at present have not clinically proved successful in fulfilling expectations despite their high cost. Some advances have been made in the control of some of the cancer types, but major deficiencies for improvement remain. In view of this, there is an urgent need to find an alternative source of therapy against this deadly disease. The phytochemicals are safer and affordable and they exhibit potent anticancerous activity (Desai & Qazi, 2008). Much research has been done on the evaluation of plant extracts as prophylactic agents with potential to inhibit cancer. The medicinal plants have been used in traditional "Ayurveda" medicine, but only a small number of these have been investigated scientifically for their potential anticancer effects (Nawab & Yunus, 2011). More than 50% of the drugs used at present to treat cancer have been isolated from natural products. The plants used include *Sausurrea costus*, *Taxus baccata*, *Cannabis sativa*, and *Podophyllum lignans* among others. In fact there are over 3000 plants reported to show anticancer activity, but the incidence of plant-derived products for cancer treatment is approximately 10%−40% (Solowey & Lichtenstein, 2014). Herbal medicines are also recognized to have high-quality immunomodulatory features and act by stimulating both nonspecific and specific immunity (Madhuri & Pandey, 2009).

The Himalayas are home to more than 60% of the total medicinal plants of the countries lying within this range with over 3000 species indigenous to Kashmir valley (Kala, 2005; Kaul, 1997; Malik, Siddique, Sofi, & Butola, 2011; Pei-Gen & Shan-Lin, 1986). The people in the Himalayan region have mastered and exercised the usage of medicinal plants which they used to grow in their close vicinity for treating various ailments through trial and error. The cherished ancient knowledge usually has been propagated through the oral messages, but now is required to be documented imperatively. During the last few decades some studies have been carried out to document the medicinal characteristics

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of plants growing in the region, particularly in the remote and difficult terrains (Haq, 2004; Kaul, 1997; Yuan, Ma, Ye, & Piao, 2016). The Kashmir Himalayas part—often referred to as a terrestrial paradise on earth—forms the northwestern tip of the Himalayan biodiversity hotspot. The valley has a rich biodiversity of plants, the majority having great medicinal value (Malik, Khuroo, Dar, & Khan, 2011). Only 500 taxa are reported from Jammu and approximately 900 from Ladakh, whereas Kashmir records around 3000 taxa (Dar et al., 2017). Currently a significant percentage of people in countries where Himalayas are distributed, especially tribals and nomads are wholly and solely dependent on traditional medicines as far as their health problems (Baig, Ramamoorthy, & Bhat, 2013; Rashid, 2012). In this chapter an attempt has been made to provide information on some medicinal and aromatic plants found in the Himalayan region. The emphasis has been laid preferably on the apoptotic and anticancer potential of these plants.

11.2 Geographical and climatic features

The Himalayan massif mountain system, including Karakoram, Hindu Kush, and some lesser ranges extending up to Pamir Knot, separates the Indian subcontinent from the Tibetan Plateau (Fig. 11.1). It is home to the highest peaks on our planet and includes Mount Everest and K2. The arc-shaped 2400-km-long main range runs from the Indus to the Brahmaputra river valley, varying in width from 400 km in the western Kashmir−Xinjiang region to 150 km in eastern Tibet (Abbasi, Khan, Ahmad, & Zafar, 2012). It encompasses about 15,000 glaciers, including the 70-km-long Siachen Glacier at the India−Pakistan border; the second-longest glacier in the world outside the polar region. These glaciers store approximately 12,000 km^3 of freshwater. The Ganges, Indus, Brahmaputra, Yangtze, Mekong, Salween, Red River (Asia), Xunjiang, Chao Phraya, Irrawaddy River, Amu Darya, Syr Darya, Tarim River, and Yellow River rise in the Himalayas (Abbasi et al., 2012).

The climate is tropical at the base of the mountains. At higher elevations we find permanent ice and snow. Annual rainfall increases from west to east. The varying altitudes, rainfall, and soil conditions generate a rich biodiversity. The xeric northwestern thorn scrub forests occupy the plains of Pakistan and the Indian Punjab, whereas moist deciduous forests lie further east around the Upper Gangetic plains, and moist deciduous forests in the Lower Gangetic plains, followed by drought-deciduous tree-dominated monsoon forests (Abbasi et al., 2012).

11.3 An overview of the plant diversity

The plant wealth varies with geomorphological features including elements from tropical Indochina, temperate East Asia, the Palaearctic region, the Deccan Plateau, and the low-lying areas, along with the support of mixed evergreen

FIGURE 11.1 Map showing the Himalayas. *Modified from Zurick, D., Pacheco, J., Shrestha, B., & Bajracharya, B. (2005).* ATLAS of the Himalaya*. Kathmandu, Nepal: International Centre for Integrated Mountain Development (ICIMOD), Hill Side Press.*

forests. Alluvial grasslands and savannas along the foothill valleys are among the high altitude on earth. Characteristic grassland species are *Saccharum spontaneum*, *Phragmitis kharka*, *Arundo donax*, *Imperata cylindrica*, *Erianthus ravennae*, *Andropogon* spp., and *Aristida ascensionis*. The slopes above 1000 m are cooler and less drought-stressed, dominated by subtropical evergreen broadleaf forests. The temperate forests in the lower reaches are dominated by evergreen broadleaf trees and mixed conifers and by winter-deciduous broadleaf species in the upper reaches. The drier, south slopes are covered by arboreal *Rhododendron* species together with *Quercus semecarpifolia* in some places or *Lyonia ovalifolia*, an ericaceous species. The temperate forests also show a rich epiphytic community from different plant groups. In the undisturbed areas *Arundinaria* spp. are dominant, where it provides early successional ground cover following fire. The altitudes between 3000 and 4000 m are covered by subalpine conifer forests, dominated by *Tsuga*, *Picea*, or *Larix* between 3000 and 3500 m in the eastern Himalayas, and above 3500 m *Abies* species are dominating. Along the treeline we come across junipers which generally are dwarf above 4500 m. The basic limestone soils support *Pinus* and *Cupressus* on dry slopes and inner valleys. The vegetation is a moist alpine scrub community above the treeline with dense juniper and *Rhododendron*s, extending up to 4500 m followed by the alpine meadows from 4500 to 4700 m, with diverse taxa of alpine herbs and dwarf woody shrubs like *Rhododendron*s, and alpine herbs like *Potentilla*, *Ranunculus*, and *Saussure* species. The high alpine areas above 4700 m are covered by periglacial and subnivean communities under high winds on unstable soils with short growing seasons, permitting only specialized plants to survive, such as *Androsacea*, *Arenaria*, *Saxifraga*, *Meconopsis*, and *Primula* (Abbasi et al., 2012).

The interaction between the people and the natural system throughout history has helped in maintaining the richness of species, communities, and genetic materials at higher altitudes in this region. Unfortunately this rich biodiversity is now destroyed and depleted because of the human pressure. Understanding the indigenous knowledge of people in relation to biodiversity resource management in this region is an important issue for sustainable development of the Himalayan region (Abbasi et al., 2012; Pei, 1994).

11.4 Important potential anticancer wild plants

Cancer is a fatal disease second only to cardiovascular diseases on a global basis (Imadi, Mahmood, & Gul, 2018; Jackson, 2000). Herbal medicines constitute the most valuable source showing potent effects against this disease (De Cicco et al., 2018; Lal, Parasar, Singh, & Akhtar, 2018; Newman & Cragg, 2016; Yuan et al., 2016). A large volume of published data has focused on the induced cytotoxicity of the alkaloids like vinblastine, topotecan, taxol, vincristine, and vinflunine, all used clinically in cancer therapy around the globe (De Cicco et al., 2018; Habli, Toumieh, Fatfat, Rahal, & Gali-Muhtasib, 2017).

11.4.1 *Acacia nilotica* (babul)

Different parts of this plant are rich sources of many phytocompounds and of these tannins like ellagic acid, gallic acid and tannic acid, vitamin C, and carotenes are known for their anticancerous activities. In male albino rats, leaf extract of *A. nilotica* has shown anticancer and antimutagenic activity in dimethylbenz (a) anthracene (DMBA)-induced skin papillomagenesis as compared to the extract from flower and gum. There is a decrease in the number of visible warts/papillomas and tumor incidence while the cytogenetic study of a bone marrow specimen has revealed a reduction in both chromatid breaks and chromosome breaks, acentric fragments and exchanges, suggesting the prominent role of *Acacia* in alleviating cancers (Meena et al., 2006). *Acacia* extract has shown an anticancerous response in intraperitoneal treatment in Dalton's ascitic lymphoma-induced solid and ascitic tumor mouse models. There was a decrease in the serum concentrations of enzymes like SGPT, SGOT, ALP, and GGT, restoration of hemoglobin and WBC levels, increased life span, and a decrease in serum levels of nitric oxide (NO) (Sakthive, Kannan, Angeline, & Guruvayoorappan, 2012). γ-Sitosterol, a phytocompound from *A. nilotica* has led to apoptosis and G2/M phase arrest of cell cycle in the breast (MCF) and lung (A549) cell line with a downstream decrease in the expression of c-Myc gene (Sundarraj et al., 2012). Acetone and methanol extracts of this plant cause significant anticarcinogenic activity in MDA-MB-231 and HEp-2 while no effect occurs in normal vero cells (Revathi et al., 2017). Polyphenols, namely ethyl gallate present in ethanolic extract of *Acacia*, are able to cause cytotoxic and anticancer activity in the cervical cell line, HeLa (Kalaivani, Rajasekaran, & Mathew, 2011).

11.4.2 *Achillea millefolium* (yarrow)

Aerial parts when put to chloroform extraction exhibit high antitumor activities on HeLa and MCF-7 cells, and a moderate effect on A431 cells. On fractionation of the extract it was found that phytocompounds like casticin and paulitin

were also highly effective against all three tumor cell lines with IC_{50} of 1.286−4.76 μm, however, apigenin, luteolin, and isopaulitin compounds have less antitumor activity with comparatively higher IC_{50} ranging from 6.95 to 32.88 μm (Csupor-Löffler et al., 2009). Selective cytotoxicity is induced in six types of human cancer cell lines, that is, human gastric adenocarcinoma, MCF-7 breast ductal carcinoma (MCF-7), colorectal adenocarcinoma (SW742), lung carcinoma (SKLC6), melanoma (A375), and hepatoma (PLC/PRF/5) (Ghavami, Sardari, & Shokrgozar, 2010). Ether extract of *A. millefolia* shows potent antiproliferative with IC_{50} values of less than 50 μg/mL in the cervical carcinoma cell line, HeLa encouraging the use of this plant for cancer chemoprevention (Bozkurt-Guzel, Serbetci, & Kultur, 2018).

11.4.3 *Achyranthes aspera* (Prickly Chaff Flower)

The roots contain triterpenoid saponins and are the most effective part although the whole plant has been evaluated medicinally (Imadi et al., 2018). The plant has been in use in India since the historic times as a traditional herb (Bagavan, Rahuman, Kamaraj, & Geetha, 2008; Imadi et al., 2018). This taxon shows antitumor, antibacterial, antifertility, and antiinflammatory activity (Imadi et al., 2018). The plant, either alone or in combination, is reported to serve as a treatment for pancreatic cancers as well as solid tumors (Subbarayana et al., 2010). According to Chakrabortya et al. (2002), during the process of carcinogenesis this plant has an inhibitory effect on tumor promoter (12-*O*-tetradecanoylphorbol-13-acetate) in Raji cells. Methanolic extract (MeOH) containing alkaloids, nonalkaloids, and saponin fractions on Raji cells has shown an antitumor effect with 76% inhibition in tumor formation. Ecdysterone and dihydroxyketone as the nonalkaloid fraction are the most effective, with 96.9% (at 100 mg/mL) anticarcinogenic activity (Chakrabortya et al., 2002). Antiproliferative activity on cancer cell lines related to pancreas, prostrate, lung, and colon has been observed with leaf extract, with effectiveness being time- and dose-dependent; pancreatic cancer cells are the most sensitive to the leaf extract. Both angiogenic and metastatic genes are inhibited by the leaf extract. For the successful survival of cancer cells two vital genes are required; pancreatic cancer cells perish through the prevention of tumor enlargement and metastasis (Imadi et al., 2018; Subbarayana et al., 2010).

11.4.4 *Aegle marmelos* (bael)

Methanolic extract of *A. marmelos* fruits has shown cytotoxic effects on human breast adenocarcinoma cells (SKBR3) as reported by Moongkarndi, Kosem, Luanratana, Jongsomboonkusol, and Pongpan (2004). According to Jagetia, Venkatesh, and Baliga (2004), Agrawal, Jahan, and Goyal (2011) and Baliga et al. (2018) chemically induced skin carcinogenesis is prevented with this extract and also the ill effects of ionizing radiation in mice are prevented.

11.4.5 *Agrimonia pilosa* (hairy agrimony)

Roots of this plant are full of tannins and have been used for the treatment of cancer (Imadi et al., 2018; Miyamoto, Kishi, Murayama, Furukawa, & Koshiura, 1988; Wang & Jin, 2011). Koshiura, Miyamoto, Ikeya, and Taguchi (1985) have undertaken studies on the methanol extract of roots of this plant. Their in vivo studies on the effects on murine syngeneic and allogenic tumors have revealed that the extract works via macrophage stimulation, in turn activating cytotoxic lymphocytes. This enlightens the fact that tumor premedication with methanolic extract is more effective postmedication. *A. pilosa* is well known for its antitumor activity, and is even used currently in China (Sugi, 1977).

11.4.6 *Anagallis arvensis* (scarlet pimpernel)

The main phytochemicals in this plant are flavanoids, saponins, terpenoids, arvenins, and tannins. In vitro studies show that a dose-dependent growth inhibition occurs in cancer cell lines, such as COLO-205, SW-620, HOP-62, T47D, DU145 IGR-OV-1, PC-3 and 786-0, by treatment with alcoholic extracts of *A. arvensis* (Agrawal, 2012). In vivo anticancer studies show that there is decrease in tumor growth in mice models induced with Ehrlich ascites carcinoma, sarcoma-180 (solid), and lymphoid leukemia (Agrawal, 2012). Apoptotic changes like activation of caspase-9, -3, and -6, cell shrinkage, mitochondrial depolarization, increased concentration of cytochrome c in cytosol, downregulation of Bcl-2, condensation of nuclear chromatin, and nuclear fragmentation are observed in HL-60 cells treated with *A. arvensis* extracts.

11.4.7 *Andrographis paniculata* (king of bitters)

Antitumor activity has been reported due to diterpene lactone present in the leaves and stem of this plant together with andrographolide. The latter is effective in oral carcinoma, interacting with several pathways (Leong, Kong, & Chung, 2018; Yang et al., 2017).

11.4.8 *Aphanamixis polystacha* (rohituka)

A. polystachya bark shows protective action and contains a compound called rohitukine (flavopiridol) which according to Arguello et al. (1998) has apoptotic effects in xenografts of human hematopoietic tumors HL-60, SUDHL-4, and Nalm/6 after being administered intravenously (Dhanamani, Devi, & Kannan, 2011; Imadi et al., 2018). The plant also has a triterpene acid—amooranin—which inhibits breast and cervical cancer proliferation by arresting cell cycle at the stage of G2/M and inducing apoptosis as mentioned by Govind (2011) and Imadi et al. (2018). The compound from the bark cited above inhibits tumor necrosis factor induced by carcinogens. TNF-induced activation of MAPK is inhibited together with a suppression of cell survival kinase (Imadi et al., 2018; Takada, Sethi, Sung, & Aggarwal, 2008).

11.4.9 *Arisaeama jacquemontii* (Jacquemont's Cobra Lily)

Lanost and norlanost are the two novel terpenoids identified in *A. jacquemontii*. Ariseminone isolated from this plant has anticancer activity (Rastogi & Mehotra, 1990). Lectins from this plant have shown an excellent antiproliferative effect on various human cancer cells, such as HCT-15, HOP-62, SW-620, HT- 29, IMR-32, SKOV3, Colo-205, PC-3, HEP-2, and A549, in vitro as observed by sulforhodamine B (SRB) assay (Kaur et al., 2006). NMR technique has found cytotoxic compounds, namely 2-hydroxydiplopterol, in chloroform extracts of this species that induce cytotoxicity in K562 cell line with IC_{50} value of 22 μM (Tanveer, Sims, Choudhary, & Hamann, 2013).

11.4.10 *Asparagus filicinus* (fern asparagus)

Saponins derived from asparagus lead to the induction of cytotoxicity and apoptosis in HCT-116 cells of colon carcinoma. Downregulation of cyclins D, E, and A cause cell cycle arrest in G0/G1. The signaling cascade components like ERK, p70S6K (mTOR), and AKT are also inhibited (Jaramillo et al., 2016). There is activation of the proapoptotic pathway of caspase-9 and caspase-3, Bax, and CytC, and downregulation in the expression of Bcl2 in cell lines on treatment with *Asparagus* saponins for 24 h in the human hepatoma cell line HepG2 (Ji, Ji, Yue, & Xu, 2012). A steroidal saponin—aspafilioside B—extracted from this plant upregulates H-Ras and N-Ras leading to ERK and p38-MAPK signal transduction activation, and ultimately to G2 arrest in human hepatoma HepG2 cells (Liu et al., 2016).

11.4.11 *Bacopa monnieri* (pennell)

It has dose-dependent anticancer effects (Imadi et al., 2018). Dalton's lymphoma cells treated with whole plant ethanolic extract have shown cytotoxicity at 150 μg/mL concentration, and oral administration reduces the risk of solid tumor formation (Imadi et al., 2018).

11.4.12 *Berberis vulgaris* (European barberry)

The alkaloid "Berberine" is found in the *Berberis* species. It elicites multiple pathways in suppressing neuroblastoma cells. A suppression of cell stemness has been seen through β-catenin and Notch (Leong et al., 2018; Naveen, Gaikwad, & Agrawal-Rajput, 2016).

11.4.13 *Cannabis sativa* (marijuana)

This plant contains tetrahydrocannabinol which has been used during the last two centuries for treating patients getting radiation or chemotherapy treatments. The side effects of these treatments are loss of appetite, cachexia, nausea, and vomiting, which according to Lal et al. (2018) are eased by these compounds. The cannabinoid receptors in the gastrointestinal system seem to be involved in inhibiting cell proliferation of colorectal carcinoma (Lal et al., 2018).

11.4.14 *Castanea sativa* (chestnut)

Glucuronoxylan, namely 4-*O*-methylglucuronoxylan, purified from chestnut is known to show anticancerous activity. It inhibits the proliferation and metastasis of A431 human epidermoid carcinoma cells with IC_{50} value of 50 μM by decreasing the release of metalloproteinases MMP-2 and MMP-9, making it a potent drug for cancer therapy (Moine et al., 2007). The T-lymphocyte cell line, Jurkat, is significantly inhibited in a dose- and time-dependent manner by activation of proapoptotic caspase-8 when treated with bark extracts of this plant (Lenzi, Malaguti, Cocchi, Hrelia, & Hrelia, 2017). Extracts of chestnut leaf are helpful in combating drug resistance which occurs against many anticancer drugs in the cancer stem cells. Inhibition of the expression of NF-E2-related factor 2 (Nrf2)—a transcription factor which helps in antioxidation and thus growth in cancer cells—occurs by chestnut extracts in breast cancer stem cells leading to antitumor activity (Woo, Oh, & Kim, 2017).

11.4.15 *Centella asiatica* (asiatic pennywort)

This plant plays a promising role in hepatoprotection, antioxidation, neuroprotection, cognition, as well as antiinflammatory and antidiabetic activity, and in wound healing. Its anticancer activity is of interest to researchers around the world (Imadi et al., 2018). Pentacyclic triterpenoid asiatic acid (AA) is the main triterpene in the plant extract with an anticancer effect against skin, human breast, gastric, and uterine cancer cells (Imadi et al., 2018; Park, Bosire, Lee, Lee, & Kim, 2005; Yoshida et al., 2005). AA possesses many pharmacological properties, including decreasing tumor cell proliferation in Ehrlich ascites tumor cells (EAC) and Dalton's lymphoma ascites tumor cells (DLA) by decreasing DNA synthesis with no toxic effects on normal lymphocytes (Babu, Kuttan, & Padikkala, 1995). The phenols like asiaticoside and madecassoside are known to inhibit cell proliferation in lung cancer cells (A549) to almost 40% (Aizad, Khairiri, Yahaya, & Zubairi, 2017). TUNEL assay has shown an increase in nuclear condensation, mitochondrial membrane disruption, and DNA breaks in the MCF7 cell lines indicating apoptosis when treated with methanolic extracts of this plant (Babykutty et al., 2009). The inhibition and cyctoxicity in the human HepG2 cell line is dose dependent and also depends on the type of treatment. If juice of this species is used at concentrations above 0.1% the activity leads to apoptotic cell death (Hussin, Eshkoor, Rahmat, Othman, & Akim, 2014). AA extracts lead to attenuation of oxidative stress and histomorphological changes in rats induced with 1,2-dimethylhydrazine (DMH) colon carcinogenesis (Siddique, Mani, Arivalagan, Thomas, & Namasivayam, 2017). AA from *C. asiatica* also induces apoptosis and cell death in lung cancer cell lines by a significant decrease in the expression of proliferating cell nuclear antigen (PCNA) and p62 gene with an increased expression of microtubule-associated protein 1 light chain 3 (LC3) (Wu, Geng, Guo, Gao, & Zhu, 2017).

11.4.16 *Cichorium intybus* (coffeeweed)

Many bioactive compounds like sesquiterpene lactones, caffeic acid, hydroxycoumarins, flavonoids, alkaloids, steroids, terpenoids, and coumarins are present in this plant (Hamid et al., 2015). Methanolic extract of chicory roots decreases cell viability of SKBR3 breast cancer lines, probably via lipid/fatty acid synthase enzyme inhibition (Mehrandish, Mellati, Rahimipour, & Nayeri, 2017). The leaves contain biologically active compounds like phenolics which have potent antioxidative actions and may make this plant an essential source for the production of healthy food as well as beneficial drugs (Jancic et al., 2017).

11.4.17 *Cimicifuga foetida* (foetid bugbane)

This plant affects menstrual and menopausal symptoms and exhibits immunomodulatory, antiinflammatory, and antimicrobial activity. The cancer prevention mechanism of this species is less well-known. A novel triterpenoid—cycloartane—extracted from this plant induces significant apoptosis and cell death by increasing autophagy in human colon cancer HT-29 cells (Dai et al., 2017). An increase of cells in G2/M phase, increase in levels of caspase-8 and -3, and poly (ADP-ribose) polymerase (PARP) occurs in the HT-29 cells. Autophagy is induced probably by increased expression of green fluorescent protein-light-chain 3 (LC3) as observed by fluorescent microscopy. According to Zhou et al. (2017), actein, a tetracyclic triterpenoid from this species, significantly enhances apoptosis in the human leukemia cell line of U937 and the primary human leukemia cells with a concomitant activation of Bax, caspase-9, caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage, and cytochrome c (Cyto-c) release. Against this, the antiapoptotic signals, like Bcl-2, Bad, protein kinase B (AKT), and ROCK1 activation, decrease leading to enhancement of

anticancerous activity. Actein from rhizomes of *C. foetida* has shown the prevention of hepatobiliary cancer by increasing proapoptotic signal transduction molecules like Cyclin Ds, p-53, and cancer stem cell markers of CD133 and a reduction in Hif-1α and VEGFR1 molecules in cancer cell lines (Xi & Wang, 2017).

11.4.18 *Coriandrum sativum* (coriander)

This plant is rich in essential oils like petroselinic acid, linalool, palmitic, and linoleic acids; minerals like potassium, calcium, magnesium, and zinc; anthocyanins and vitamin A. All these show a wide range of nutritional and pharmacotherapeutic characteristics. Coriander shows a high degree of free radical scavenging. Its root extract using ethyl acetate as solvent causes antiproliferative action on MCF-7 at the same time protecting DNA damage in normal cells (Tang, Rajarajeswaran, Fung, & Kanthimathi, 2013). There is arrest of cell division in G_2/M phase and apoptosis by activating death receptor and mitochondrial apoptotic pathways in breast cancer cells. The anticancer and antioxidative activities in this plant are due to the presence of vitamin C in the roots (Tang et al., 2013). Nithya and Sumalatha (2014) have reported a decrease in cell viability and a concomitant increase in dead cell count is seen in human colon cancer (HT-29) cell lines using ethanolic extract from its leaves, but the mechanism and signaling for this anticancer activity is not yet established.

11.4.19 *Crataegus* species (hawthorn)

The plant shows immense free radical scavenging and antiinflammatory activity. Corsolic acid derived from this plant is known to have significant cytotoxic action against a number of human cancer cell lines and a dose-dependent inhibition in the protein kinase C (PKC) activity of leukemic cells (Ahn, Hahm, Park, Lee, & Kim, 1998). The triterpenoid enriched fraction of the plant causes downregulation of PCNA, Cyclin D1, and CDK4 and upregulation of p21 leading to antiproliferation in MDA-MB-231, $HepG_2$, and MCF-7 cell lines, as reported by Wen et al. (2017). Its ursolic acid fraction shows potent antiproliferative action in cancer cell lines. According to Rodrigues et al. (2012) phenolic acids from flower buds are mainly composed of hydroxycinnamic acid which shows antiproliferative action in various human cancer cell lines like breast adenocarcinoma (MCF-7), nonsmall cell lung cancer (NCI-H460), cervical carcinoma (HeLa), and hepatocellular carcinoma (HepG2). Ethyl acetate extract of leaves causes apoptosis and cell cycle arrest in the G1 stage with prominent DNA fragmentation, decrease in Bax levels, and cleavage of poly (ADP-ribose) polymerase (PARP) in HT-29 and HCT-116 human colorectal cancer cells in a dose-dependent manner (Mustapha et al., 2016).

11.4.20 *Curcuma longa* (turmeric)

The major component present in this plant is a polyphenol diferuloyl-methane, commonly known as curcumin, extracted from the rhizome; a hydrophobic compound which gives it the characteristic yellow color (Huang, Smart, & Wong, 1998; Imadi et al., 2018; Oetari, Sudibyo, Commandeur, Samhoedi, & Vermeulen, 1996; Singh & Khar, 2006). This compound includes curcumin I, curcumin II, curcumin III, and curcumin I (diferuloyl-methane) (Imadi et al., 2018; Rubya, Kuttana, Babub, Rajasekharanb, & Kutta, 1995). Curcumin works in different ways in the inhibition of tumor formation and cancer progression. Several mechanisms of have been proposed in this connection. Possible mechanisms effective against cancer have been studied at length (Imadi et al., 2018).

Anticancer activity of rhizomes has been investigated for a long time using Dalton's lymphoma cells in ascites form (Kuttan, Bhanumathy, Nirmala, & George, 1985), and curcumin has shown cytotoxicity in lymphocytes and Dalton's lymphoma cells in vitro. It has also been found to inhibit the development of animal tumors in several experimental studies and is currently being developed as an anticancer drug (Imadi et al., 2018; Kelloff et al., 1996).

Turmeric is also effective on human colon cancer cells, inhibiting the proliferation of HT-29 and HCT-15 (Hanif, Qiao, Shiff, & Rigas, 1997). According to Singh, Sidhu, Deepa, and Maheshwari (1996), in tumor development cyclooxygenase-2 (COX-2) plays an important role. The colon carcinogenesis is inhibited via a decrease in the colon tumors by curcumin (Imadi et al., 2018).

Curcumin also inhibits the proliferation of androgen-dependent and androgen-independent prostate cancer cells. There is a suppression of colonic aberrant crypt focus formation in skin, stomach, and colon cancers due to this effective chemopreventive compound (Imadi et al., 2018). It works best in its demethylated form as methylation affects its antioxidant and antitumor activities (Imadi et al., 2018).

11.4.21 *Cuscuta reflexa* (dodder)

Different dodder species are known to have several therapeutic effects, such as anticancer, immunostimulatory, hepatoprotection, antioxidant, and antiosteoporotic activity. *C. reflexa* extract in water causes apoptotic cell death in Hep3B cells by upregulating proapoptotic factors BAX and p53, and downregulating the expression of antiapoptotic factors Bcl-2; there is also a decrease in the expression of inflammatory mediators like COX-2 and TNF-α genes (Suresh, Sruthi, Padmaja, & Asha, 2011). A dose- and time-dependent inhibition of Hep 3B cell line proliferation is seen on treatment with the chloroform extract of this plant by activating caspases, DNA fragmentation, PARP cleavage, and arrest in the G1 stage (Praseeja, Sreejith, & Asha, 2015). Administrating its chloroform and ethanol extracts in albino mice injected with EAC cells causes a significant decrease in tumor volume and viable cell count, in turn increasing the life span of animals suffering from cancer (Chatterjee, Sahu, Jha, & Dwivedi, 2011). Scoparone, *p*-coumaric acid, stigmasta-3,5-diene, and 1-*O*-*p*-hydroxycinnamoylglucose bioactive compounds show promising antitumor activity against colorectal cancer cell lines (HCT-116) (Riaz et al., 2017).

11.4.22 *Cynodon dactylon* (Bermuda grass)

The extracts of this grass are known to have several bioactive molecules like hydroquinone levoglucosenone, and furfural, which make it a potent drug for diabetes, cancer, liver, and several inflammatory disorders as well as wound healing. According to Kanimozhi and Bai (2013) its ethanolic extract produces cytotoxicity in the HT-29 human colon cancer cell line comparable to the synthetic drug cyclophosphamide and is nontoxic to the vero cells as well. Anthocyanins like delphinidin, petunidin, malvidin, cyanidin, and their various derivatives are reported to possess significant inhibitory effects on MCF-7 (Khlifi et al., 2013). Due to prominent hepatoprotection and maintenance of antioxidant status, methanolic extracts of roots of this plant are able to help in alleviating hepatocellular carcinoma produced by diethyl nitrosamine in rats (Kowsalya, Kaliaperumal, Vaishnavi, & Namasivayam, 2015). Its methanolic extracts also show anticancer and antioxidative activities in the colon carcinoma cell line COLO 320 by inducing apoptotic cell death in these cells (Albert-Baskar & Ignacimuthu, 2010).

11.4.23 *Elaeagnus angustifolia* (Russian olive)

This species is a good source of glycosylated quercitin flavonoids, glycosylated kampferols, catechins, epicatechins, sterols, and alkaloids with potential anticancer and antioxidative potential. Most potent antioxidant activity is seen in the water/ethanol (1:1) extract of leaves, as well as phenolics in these plants, which help them to act as potent scavengers of free radicals. According to Badrhadad, Kh, and Mansouri (2012) hydroalcoholic extracts have antiangiogenic effects in the endothelial cells of umbilical vein in humans, thereby protecting cells from tumor expansion and metastases. In addition it contains many essential oils like ethyl cinnamate, phenylethyl benzoate, phenylethyl isovalerate, nerolidole, acetaphenoneand squalene, flavonoids, and proanthocyanosides all with anticancer activity (Wang, Fan, Li, & Guo, 2013). Significant improvement in the morphology of rats is seen histopathologically following induction with ulcerative colitis and then applying edible extracts of this plant (Khodakarm-Tafti, Mehrabani, Homafar, & Farjanikish, 2015). Extract of this species shows significant antiproliferative activity against HeLa cells (Ya et al., 2014).

11.4.24 *Euphorbia helioscopia* (sun spurge)

The leaves of this plant are rich in quercitin which has potent anticarcinogenic properties. The report published by Wang et al. (2012) has revealed that ethyl acetate extract from this plant decreases the proliferation rate of five human cancer cell lines: three hepatocarcinoma cell lines (SMMC-7721, BEL-7402, HepG2), one gastric carcinoma cell line (SGC-7901), and one colorectal cancer cell line (SW-480). The highest inhibition has been recorded in the proliferation of SMMC-7721 cell line after treatment with the extract of this plant. The treated SMMC-7721 cell lines have shown apoptotic bodies, changes in morphology, arrest of cell cycle in G1 phase, decrease in tumor cell progression, and inhibition of MMP-9 expression. Aqueous extract of the roots of this species used with IC_{50} values of 1.26, 1.98, 1.72 mg/mL, respectively, has led to tumor inhibition in SMMC-7721, HeLa, and MKN-45 cells, and the roots increase survival rate in S180 and H22 tumor-bearing mice by improving the immune response (Cai, Lu, Liang, Yu, & Xie, 1999; Cai, Wang, & Liang, 1999). Euphornin—a macrocyclic diterpenoid present in this plant—exhibits significant cytotoxicity against HL-60 cell lines (Tao, Hao, Liu, & Zhu, 2008). In the mouse xenograft model of hepatocellular carcinoma a downregulation in the expression of proteins like CyclinD1, bcl-2, and MMP-9 and a simultaneous upregulation of bax,

caspase-3, and nm 23-H1 proteins is seen after treatment with the ethyl acetate extract of *E. helioscopia* depicting apoptosis and antitumorogenesis (Cheng et al., 2015).

11.4.25 *Euphorbia tirucalli* (fire sticks)

Many phenolics and terpenes have been identified in the extracts from the photosynthetic stems following chromatographic and spectroscopic analyses, the most important being triterpenes, euphol, and tirucallol (Nizami & Sayyed, 2018). A key factor in combating cellular oxidative stress is the antioxidant activity found in the leaf/stem extracts. The whole plant methanol extract of this species has positive antioxidant activity. Its latex is also used in traditional medicine to treat cancer and this has attracted much interest in many advanced countries (Harborne & Williams, 1992; Nizami & Sayyed, 2018; Stray & Storchova, 1991).

11.4.26 *Mangifera indica* (mango)

It is one of the important fruits of India, and also one of the most liked fruit crops of Asia as well as other tropical and subtropical regions of the world (Baliga et al., 2018; Morton, 1987). Mangiferin is a principal constituent of the fruit and has protective effects against chemical-induced bowel−lung carcinogenesis, UV-induced skin damage, and deleterious effects of ionizing radiation (Baliga et al., 2018; Jagetia & Baliga, 2005; Lei et al., 2012; Petrova, Davids, & Rautenbach, 2011; Rajendran, Ekambaram, Magesh, & Sakthisekaran, 2008; Rajendran, Ekambaram, & Sakthisekaran, 2008a, 2008b, 2008c; Yoshimi et al., 2001). The anticancer activity is also due to the presence of carotenoids, lupeol, gallic acid, caffeic acid, quercetin, and kaempferol present in the fruit (Baliga et al., 2018). Percival et al. (2006) have also reported some anticancer activity for mango juice, which reduces the number of transformed foci and arrests some of the phases.

11.4.27 *Matricaria chamomilla* (German chamomile)

Many important bioactive compounds, such as coumarins, for example, herniarin, umbelliferone; phenylpropanoids like chlorogenic acid, caffeic acid; flavones, for example, apigenin, apigenin7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside; flavonols like quercetin, rutin, and flavanone naringenin have been found in chamomile extract. The major constituent of aqueous and methanolic extracts is apigenin 7-*O*-glucoside which shows differential apoptosis in many human cancer cells but not in normal cells at similar doses. Very few studies have been carried out on the anticancer activity of chamomile (Srivastava & Gupta, 2007).

11.4.28 *Momordica charantia* (bitter melon)

The bitter gourd seeds contain alpha steric fatty acid (ASFA) in high quantity which can lead to growth inhibition of cancer cells. Its hot water extract decreases mammary tumor growth when fed to mice in drinking water, however, the mechanism for this inhibition is as yet unknown (Nagasawa, Watanabe, & Inatomi, 2002). According to Ray, Raychoudhuri, Steele, and Nerurkar (2010) its extracts induce apoptosis and G_2-M phase cell cycle arrest of breast cancer cells by increasing poly (ADP-ribose) polymerase cleavage, expression of p53, p21, and pChk1/2, and caspase activation with a simultaneous inhibition of surviving cyclin B1, cyclin D1, and claspin expression. Positive results have been observed in the case of prostrate cancer cells when PC-3 and LNCaP are treated with BME which accumulates during the S phase with increased expression of cyclin D1, Bax, cyclin E, and p21 and PARP cleavage (Ru, Steele, Nerurkar, Phillips, & Ray, 2011). Prostatic intraepithelial neoplasia in TRAMP (transgenic adenocarcinoma of mouse prostate) mice has decreased in progression when bitter melon extract is given orally. Its seed oil is a nutritional oil enriched with many medicinally important compounds with more than 60% being linoleic acid. According to Yasui et al. (2005) this oil induces a dose-dependent apoptosis in Caco-2 by decreasing the expression level of apoptotic suppressor Bcl-2 protein with a concomitant upregulation of GADD45, p53, PPARγ mRNA, and protein expressions. The bitter gourd extract given orally to the mice with 4-nitroquinoline 1-oxide (4-NQO)-induced head and neck squamous cell carcinoma (HNSCC) significantly prevents cancer formation (Sur et al., 2017). Neoplastic changes in histopathology with an increased expression of proinflammatory genes, s100a9, IL23a, IL-1beta, and PDCD1/PD1, may be the signaling mechanisms for preventing oral cancers after bitter gourd treatment.

11.4.29 *Narcissus tazetta* (bunchflower daffodil)

N. tazetta possesses antimicrobial, anticancerous, antiinflammatory, and antioxidative pharmacological activities. Liu, Li, Ren, and Hu (2006) have reported that the extracts applied to the cancer cell line HL-60 cause apoptosis and cell death by release of cytochrome c and upregulation of caspase-8, -9, and -3 and Bax gene expression. Pseudolycorine—an alkaloid from the extract of this plant—leads to an increased survival rate in mice with Rauscher leukemia via a reduction in splenomegaly (Furusawa, Furusawa, Morimoto, & Cutting, 1971). The two derivatives from narcissus with potent antitumor activity are narciclasine and narcistatin (Pettit et al., 2003). Application of chloroform extracts of the bulbs and roots of this plant have ended up with an inhibition in proliferation of a hepatocellular carcinoma cell line (HEPG2) and a colon carcinoma cell line (HCT-116) (Shawky et al., 2015).

11.4.30 *Oroxylum indicum* (India caper)

The methanolic extracts of this plant used for treatment in the HL-60 cell line has given almost 50% reduction in cell viability due to the presence of a flavonoid, namely baicalein with an increased number of cells in S or G2/M phases. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay has revealed that nuclear DNA fragmentation indicates cell death by apoptosis (Kumar Roy et al., 2007). A significant antiinflammation is mediated by NF-κB inhibition; this could therefore be a potent drug for cancer therapy. The two flavonoids present in this plant, namely, chrysin and oroxylin, show promising apoptosis-inducing ability (Khoo, Chua, & Balaram, 2010; Siriwatanametanon, Fiebich, Efferth, Prieto, & Heinrich, 2010).

11.4.31 *Oxalis corniculata* (creeping woodsorrel)

Its ethanolic extract shows anticancer and antioxidant activity in Ehrlich acsites carcinoma (EAC) induced in swiss albino mice. Kathiriya, Das, Kumar, and Mathai (2010) report that all hematological, biochemical, and antioxidant profiles are normalized with a concomitant reduction in body weight, tumor cell volume and count, and an increased survivability. Its methanolic extract has cytotoxic effects on hepatic cancer, Hep2 cell line, but no toxic effects in normal human corneal epithelial cells (HCEC) with an IC_{50} of 0.048 mg/mL (Salahuddin et al., 2016).

11.4.32 *Paeonia emodi* (Himalayan peony)

It shows potent free radical scavenging activity because of high phenolic content (Khan et al., 2005). Antinutritional agents like phytic acid and tannins are present in the extracts of this plant which seem to be responsible for DNA protection and act as antimutagenic agents as well. However, anticancer activity is still questioned (Jugran et al., 2016).

11.4.33 *Persicaria hydropiper* (syn.: *Polygonum hydropiper*) (water pepper)

Different extracts of *P. hydropiper* show anticancer activity. Ayaz et al. (2016) report that there is a decrease in angiogenesis of mouse embryonic fibroblast NIH/3T3 cell lines leading to decreased tumor metastases. The extracts lead to growth inhibition in cervix epithelial adenocarcinoma (HeLa), breast epithelial adenocarcinoma (MCF7), and skin epidermoid carcinoma (A431) cell lines (Lajter et al., 2013). However, the molecular mechanisms must be investigated.

11.4.34 *Pinus* species (pines)

Different species of the genus *Pinus* are reported to show anticancer activity against many cancer cell lines. These plants are rich in polyphenolic compounds, like taxifolin and catechin, which possess broad pharmacological activity and are marketed as picnogenol. Treatment with pycnogel is said to cause growth inhibition in the HL-60 leukemia cell line with IC_{50} of 150, 40, and 100 μg/mL respectively (Huang, Yang, Lin, Ho, & Lee, 2005). Essential oils from the leaf and bark of *Pinus eldarica* produce cytotoxic effects on both HeLa and MCF-7 cancer cell lines due to the presence of a high concentration of terpenoids and phenolics (Sarvmeili, Jafarian-Dehkordi, & Zolfaghari, 2016). Ethanol extract of the *Pinus densiflora* needles shows antitumorogenic action by inhibiting growth of KATO and MCF-7 cells with IC_{50} of 209 and 241 μg/mL, respectively (Yim, Hong, & Lee, 2006). Ultraviolet rays (UVR) with other carcinogens (7,12-dimethylbenz[a]anthracene) induce nonmelanoma skin cancers (Kyriazi, Yova, Rallis, & Lima, 2006). If bark extract is used against UVR it shows anticancer activity by decreasing tumor volume and occurrence due to its richness in phenolic acids. Procyanidins from *Pinus pinaster* are significant antiproliferative agents for melanoma cells, due to

strong free radical scavenging and antioxidative effectiveness (Touriño et al., 2005). Cui, Xie, Qi, He, and Wang (2005) have reported that apoptosis, with formation of a typical DNA ladder, and nuclear fragmentation are seen in human hepatoma (BEL-7402) cell lines in a dose- and time-dependent manner. Downregulation of the bcl-2 gene expression in these cells seems to be the possible cause of apoptosis. Essential oils from the bark, wood, and needles of *Pinus roxburghii* are reported to show antioxidant and antimicrobial activities against many human pathogens (Salem, Ali, & Basalah, 2014), however, one study has reported its anticancer activity due to its sesquiterpenes fraction which induces cytotoxicity in lung (A549), glioma (C6), breast (T47D, MCF), and colon (TH-1) cancer cell lines (Qadir, Shah, & Banday, 2014). A combined use of *Cedrus deodara* and *P. roxburghii* shows a protective effect against gastric ulcers induced in rats, by decreasing gastric hemorrhage, acidity, and maintaining tissue integrity (Chaudhary, Ahmad, & Mazumder, 2014).

11.4.35 *Plectranthus* species (coleus)

The diterpenoids and triterpenoids present as secondary metabolites in these species are medicinally important. The most abundant diterpenoids found in its species are labdanes, abietanes, and ent-kauranes (Waldia, Joshi, Pathak, & Joshi, 2011). Diterpenoids extracted from the leaves of Indian *Plectranthus coesta* are reported to show antiproliferative action against human glioblastoma cells (U87) (Waldia et al., 2013). Similarly *Plectranthus amboinicus* extracts also show potent cytotoxic action in MCF7 and T47D, mainly due to cell cycle inhibition and apoptotic cell death (Hasibuan & Nasution, 2013).

11.4.36 *Plumbago zeylanica* (Ceylon leadwort)

The metabolite plumbagin—a naphthoquinone derivative found in the roots—inhibits different cancer types following multiple pathways. It regulates proliferation, survival, invasion, and metastasis (Leong et al., 2018). This has been demonstrated to be effective in the case of prostate CSCs and breast cancer susceptibility, as well as mutated prostate cells following the triggering of intrinsic and extrinsic apoptosis (Leong et al., 2018).

11.4.37 *Portulaca oleracea* (common purslane)

P. oleracea is reported to be effective pharmacologically in the treatment of tumors, bacterial infections, wound healing, muscle relaxation, and as an antiinflammatory agent. Farshori et al. (2014) using MTT and neutral red uptake (NRU) assays have shown that a dose-dependent decrease in cell size/viability occurs after treatment of the hepatocellular cell line HepG2 with seed extracts of this plant. In the same way, the treatment of KATO III (human gastric carcinoma cell line) and COLO 320 HSR cells (human colon adenoma cell line) with the aqueous extracts of this species results in the recession of growth in gastric and colon cancers (Yoon, Ham, & Jun, 1999). However, in the case of normal cell lines like murine lung connective tissue (L929) and human lung diploid cell (W138) cells no effect has been recorded. A visible decrease in tumor growth in mice has been observed using the same extract in in vivo studies when cancer-induced nude and untreated cancer-induced mice are compared (Yoon et al., 1999). According to Shen et al. (2013), an antitumorogenic activity induced due to its immunomodulatory effect occurs due to the polysaccharide from this plant by leading to an increase in the number of immunoresponsive cells, like white blood cells (WBC) and CD4 T lymphocytes. The sulfate derivatives of these polysachharides also significantly inhibit HepG2 cells and HeLa cells, which may be attributed to the possible cell cycle arrest at S phase, indicating the sulfation of polysaccharides which probably increases the cytotoxic effect of purslane (Chen et al., 2010).

11.4.38 *Potentilla species* (cinquefoil)

Hossan et al. (2014) and Lal et al. (2018) have shown that kaempferol—a major phytochemical in *Potentilla fulgens*—affects pancreatic cancer cells by acting on the protooncogene tyrosine protein kinase (Src), Erk1/2, and Akt pathways, retarding their growth and migration. The investigations undertaken and published by Lal et al. (2018) have revealed that apoptosis in breast and prostate cancer cells is inhibited by ellagic acid found in this species which inhibits metastasis processes of various cancer types. Several useful phytocompounds have been reported from *Potentilla nepalensis*, such as flavonoids like kaempferol, leucoanthocyanidin; catechins, ellagic acids, etc., all showing antiinflammatory, antiulcer, antibacterial, antihyperglycemic, and antidiarrheal activities. Its extracts have shown significant anticancer activity due to the presence of corosolic acid and trihydroxyursenoic acid as confirmed by HPLC studies (Liu, Duan,

Pan, Zhang, & Yao, 2006). Some work has been carried out on the ethyl acetate extract of *Potentilla chinensis*. The aerial parts contain a triterpenoid which leads to DNA ladder formation (an indication of DNA fragmentation), formation of apoptotic blebs, and an arrest of cell cycle in G0/G1 phase in osteosarcoma as revealed in the in vitro cytotoxic action against SMMC-7221 human hepatoma and HL-60 human promyelocytic leukemia cells (Liu, Duan, et al., 2006).

11.4.39 *Prangos pabularia* (prangos)

P. pabularia root extract is a rich source of different phytocompounds. These show cytotoxicity against lung (A549 and NCI-H322), melanoma (A375), prostate (PC-3), epidermoid carcinoma (A431), and colon (HCT-116) cell lines of humans. Out of these bioactive compound "osthol" shows maximum antiproliferative action (Farooq et al., 2014). A treatment with the essential oils derived from some *Prangos* species has inhibited the growth of renal adenocarcinoma cell line (Loizzo, Tundis, Menichini, Saab, & Statti, 2008).

11.4.40 *Rheum webbianum* (rhubarb)

Rhubarb contains essential phytocompounds like anthraquinones, such as rhein, emodin, aloe-emodin, physcion, and chrysophanol; crysophanic acid/crysophan, a glycoside rhaponticin; tannins like sennosides, catechins, gallic acid, and cinnaminicacid. Its extracts inhibit squalene epoxidase, an important enzyme for fat metabolism, thereby producing hypocholestremic effects (Abe, Seki, Noguchi, & Kashiwada, 2000). According to Srinivas, Babykutty, Sathiadevan, and Srinivas (2007) anthraquinones found in this species help in alleviating cancer. Extracts from this plant show antitumor activity in cancer cell lines by inducing apoptosis and inhibiting cell cycle progression and angiogenesis. The extract also serves as an adjunct to chemotherapy, however, more research regarding the anticancer effects on a wider scale in this connection are needed in order to use it as a promising medicine for cancer chemoprevention.

11.4.41 *Rhodiola imbricata* (golden root)

R. imbricata is a plant rich in flavonoids, tannins, terpenes, phenyl ethanol derivatives, and phenylpropanoids. It shows significant antioxidative potential because of phytosterols, phenols, and fatty acid esters (Tayade et al., 2013). According to Choudhary et al. (2015) ethyl acetate extract of plant roots acts as an apoptogenic and antioxidative against the A549 and MCF-7 cancer cell lines—the reason being the wide range of phenolic glycosides and other phenols. Mishra, Chanda, Shukla, and Ganju (2010) have reported that extracts of this plant lead to an increased immunogenicity by acting as an adjuvant in increasing humoral as well as cell-mediated immune responses. It has been seen that *R. imbricata* aqueous extract causes a significant decrease in proliferation of the human erythroleukemic cell line K562 by inducing apoptosis indicated by cell cycle arrest at G2/M phase and cytotoxicity in NK (natural killer) cells when using MTT cell proliferation assay, but no toxic effect has been observed on normal blood lymphocytes and a mouse macrophage cell line (RAW-264.7) (Mishra et al., 2008).

11.4.42 *Saxifraga stolonifera* (strawberry saxifrage)

Chen, Liu, et al. (2008) have reported that ethanol extract of *S. stolonifera* decreases cell viability of a gastric carcinoma cell line (BGC-823) by inducing apoptosis and DNA fragmentation, which could be due to its richness in quercitin. Potential antimicrobial and antioxidant activity has been recorded for the aerial parts of this species which is due to the presence of glycosylated phenols (Sohn et al., 2008).

11.4.43 *Sida cordifolia* (bala)

S. cordifolia roots are well known and used widely in India as an ingredient for preparing various formulations of Ayurvedic medicines (Ahmed et al., 2018). Cytotoxic activity of whole plant ethanolic extracts has been observed to show dose-dependent cell toxicity in HeLa cell lines (Joseph, Ajisha, Kumari, & Sujatha, 2011). These researchers report that cells treated with the extract of this plant arrest the cell activity via apoptosis. Its 70% ethanolic extract at a dose of 250 and 500 mg/kg bw has significantly restored necrotic cells, which can be attributed to its antioxidant and anticancer activities (Ahmed et al., 2018; Mallikarjuna, Reddy, & Prabhakaran, 2013).

11.4.44 *Silybum marianum* (milk thistle)

Milk thistle has been used as liver tonic for centuries and is distributed from Southern Europe to Asia (Leong et al., 2018). There are seven major flavonolignans and a flavonoid found in the commercial product silymarin, one of these being silibinin, found as a mixture of silybin A and silybin B (Leong et al., 2018). It shows antitumor features associated with mesenchymal characteristics in nonsmall cell lung carcinomas. The reports published by Corominas-Faja et al. (2013) and Leong et al. (2018) show that silibinin reverses the erlotinib resistant cells, used to enrich CSCs in the culture.

11.4.45 *Sinopodophyllum hexandrum* (syn.: *Podophyllum emodi*) (Himalayan may apple)

The naturally occurring aryltetralin lignans—podophyllotoxin and deoxypodophyllotoxin—have been identified in this species (Srivastava, Kumar, Gupta, & Khanuja, 2005; Tan & Norhaizan, 2018). According to Tan and Norhaizan (2018) it is well-known for its use in the treatment of warts and skin cancer as well as non-Hodgkin's lymphoma, lung cancer, and genital tumors. Dried root alcohol extract contains podophyllin. It is effective against venereal warts when applied topically. The lignans have been purified and introduced into clinical trials as other podophyllotoxin compounds. However, studies were stopped due to the undesirable toxicity. After several studies conducted during the 1960s and 1970s teniposide and etoposide were developed. These are used as clinical agents in the treatment of bronchial, testicular, and lymphatic cancers. Out of more than 2000 anticancer clinical trials after the year 2004 more than 150 applications are drug combinations with etoposide for the treatment of numerous cancers (Tan & Norhaizan, 2018).

11.4.46 *Solanum nigrum* (European black nightshade)

Alkaloids, flavonoids, tannins, saponins, glycosides (solamargine, solasonine, α and β-solanigrine), proteins, carbohydrates, coumarins, and phytosterols have been identified in this plant. It shows antiinflammatory, antibacterial, antioxidant, antigastritis, and antiulcerogenic and cardioprotective effects. Fruit methanolic extract shows inhibitory effects on the growth of HeLa cell, but less effect is seen in a normal cell line (Vero) (Patel, Gheewala, Suthar, & Shah, 2009). A potent glycoside, solamargine, found in this plant causes cell death via caspases, tumor suppression, and death receptor pathways, as well as through kinases. According to Li, Li, Gao, Han, and Lu (2009) the polysaccharides of *S. nigrum* increase the proapoptotic Bax gene and decrease Bcl-2 and mutant p-53 expression. Its glycoalkaloids show antitumorogenic activity to various hepatocellular carcinoma cell lines (HepG2 cells and AGS) (Rajalakshmi and Jayachitra, 2017). Lunasin (a 43-amino acid peptide) is responsible for the chemoprevention in skin cancer found in mouse models by inhibition of acetylation of core histones (Jeong et al., 2007). Zhao et al. (2017) have reported that another compound from this species—degalactotigonin—causes growth inhibition and decreases metastasis in osteosarcoma cells through inactivation of GSK3β via the Hedgehog/Gli1 pathway. A potent saponin from this species—Uttroside B—shows good anticancer activity in hepatocellular carcinomas HepG2 cells by downregulating MAPK and mTOR pathways, as reported by Nath et al. (2016). Benzoiisovanillin and syringic acid (4-hydroxy-3, 5-dimethoxybenzoic acid) are the two important phenolics found in this plant that are responsible for wide range of pharmacological activities (Khan et al., 2016a). The berries show an antiproliferative action on the MDA-MB-231 and MCF-7 breast cancer cell lines. The polysaccharide fraction of the plant suppresses tumors in breast cancer through host immune response (Prakash, Maurya, & Ajeet, 2018).

11.4.47 *Stellaria media* (chickweed)

Not many studies have been undertaken on the anticancerous activity of this species in spite of the fact that it is a phytochemically rich plant. These phytochemicals could act as potent anticancerous agents. Its important constituents are phenolic acids, that is, vanillic acid, *p*-hydroxybenzoic acid, ferulic acid, caffeic acid, and chlorogenic acid; flavonoids, that is, apigenin and genistein; and flavones, that is, gypsogenin, a triterpenoid saponin, ß-sitosterol, and its ß-D-glucoside. Bukola and Bernard (2011) have reported that leaf methanol extract shows potent antioxidant activity and can be used against different inflammatory conditions and cancerous cells, however, the plant is toxic to lambs but cattle can eat it without any effect. Adverse toxic allergic reactions have been observed in human beings, probably due to the release of some contact allergens like borneol and additional terpenes (Jovanović et al., 2004). This species shows antiviral, antilipidemic, and antitumor activity (Sharma and Arora, 2012). There is a need for detailed studies on the chickweed for its anticancerous activity.

11.4.48 *Syzygium cumini* (jamun)

The plant has been recorded to be antidiabetic, anticancerous, antioxidant, antibacterial, antifungal, and antidiarrheal due to its richness in many compounds. Its fruit alcoholic extract contains anthocyanins which cause cytotoxicity in HeLa, breast, liver, lung, and brain carcinoma cell lines, as reported by Nazif (2007), whereas ethanolic extract is rich in phenolic compounds and sterols exhibiting antileukemia activity (Afify, Fayed, Shalaby, & El-Shemy, 2011). Oral administration of *S. cumini* significantly reduces chemical carcinogenesis induced by benzo[a]pyrene (BaP) in mice stomach, with a reduction in tumor incidence and simultaneous increase in phase II detoxifying enzymes, and inhibition of lipid peroxidation (Goyal, Verma, Sharma, Parmar, & Agarwal, 2010). Studies on the human cervical cancer cell lines with jamun pulp have revealed that crude and methanolic extracts induce a time-dependent increase in apoptosis (Baliga et al., 2018). Li, Adams, et al. (2009) have shown that equivalent jamun fruit extract concentrations induce apoptosis and inhibit proliferation of breast cell lines effectively, the fruit extract however, does not affect the nonmutagenic and normal breast cell lines (Baliga et al., 2018). It has also been reported to possess radioprotective and preventive effects in experimentally induced skin cancers (Jagetia & Baliga, 2002, 2003; Jagetia, Baliga, & Venkatesh, 2005; Parmar, Sharma, & Verma, 2011).

The findings reported by Kausar et al. (2012) have clearly highlighted the fact that a combination of suboptimal concentrations of equimolar anthocyanidins inhibits the growth of two aggressive nonsmall cell lung cancer cell lines synergistically, with negligible effects on the viability of nontumorigenic cells. These researchers have further reported that a combination of anthocyanins proves to be much more effective as an anticarcinogenic agent than the individual components. The protective ability of jamun is interfered through the alteration of oncogenic Notch and Wnt pathways by enhancing cleavage of the apoptotic mediators and increasing the inhibition of TNFα-induced NF-κB activation. Inhibition of growth following the administration of delphinidin has also been reported.

11.4.49 *Tabernaemontana divaricata* (crape jasmine)

Anticancer activity at IC_{50} with more than 100 μg/mL has been recorded with hydroalcoholic extract. The leaf ethanolic extract is cytotoxic against tumor cells and could be a candidate for chemoprevention (Dantu, Shankarguru, Ramya, & Vedha, 2012; Imadi et al., 2018; Khan & Islam, 2012).

11.4.50 *Terminalia arjuna* (arjuna)

Rich in tannins, triterpenoid saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), gallic acid, ellagic acid, oligomeric proanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc, and copper (Bone, 1996; Imadi et al., 2018; Kapoor, 1990; Pasquini et al., 2002), it has been used traditionally for treating many health problems (Imadi et al., 2018).

Some studies have been carried out on the chemical compounds from *T. arjuna* for a treatment of cancer and associated diseases. Gallic acid (GA), ethyl gallate, and flavone luteolin have been isolated from the bark, stem, and leaves of this plant (Imadi et al., 2018). Out of these, arjunic acid shows antitumor and antimutagenic activity and it also inhibits a number of ascites, solid tumors, and leukemia in cell lines (Chen, Huang, Li, Fang, & Chen, 1992; Hu, Percehellet, Klish, Johnson, & Perchellet, 1992; Imadi et al., 2018; Matsukawa et al., 1993; Molnar et al., 1981; Post & Varma, 1992; Ryu et al., 1994; Saxena et al., 2007; Sivalokanathan, Ilayaraja, & Balasubramanian, 2005).

11.4.51 *Tribulus terrestris* (goat's head)

This plant contains "spirostanol" a saponin which has cytotoxic effects against many human cancer cell lines, such as human breast ductal carcinoma (BT-549), malignant melanoma (SK-MEL), human oral epidermoid carcinoma (KB), and human ovary carcinoma (SK-OV-3) (Bedir, Khan, & Walker, 2002). The saponin fraction of this plant inhibits the proliferation and simultaneous induction of the apoptotic molecular pathway in many cancer cell lines. In general, normal human fibroblast was less affected (Neychev, Nikolova, Zhelev, & Mitev, 2007). According to Kim et al. (2011) aqueous extract of this plant inhibits HepG2 cell proliferation via the inhibition of NF-κB signaling. Its fruit extract has inhibited skin papillomas in mice (Kumar, Soni, Shukla, & Kumar, 2006).

11.4.52 *Trillium govanianum* (nag chhatri)

T. govanianum methanolic extract is rich in glycosides, steroidal saponins, tannins, sterols, and flavonoid. Khan et al. (2016b) have reported that solid-phase extraction (SPE) of methanolic extract of its roots is significantly anticancerous, showing cytotoxicity in breast (MCF7), liver (HEPG2), lung (A549), and urinary bladder (EJ138) cancer cell lines with IC_{50} values ranging between 5 and 16 μg/mL. This species contains nearly 5% of a steroidal sapogenin (diosgenin) which is used in the synthesis of steroidal and sex hormones. The hydroalcoholic extract containing diosgenin shows highly cytotoxic effects on MCF-7 and MDA-MB-231 breast cancer cells, whereas the normal cell line MDCK is affected to a lesser extent (Sharma et al., 2016). Different extracts from the rhizome of this species show antiproliferative activity and cytotoxicity in HeLa and PC-3 cell lines (ur Rahman, Ismail, Shah, Iriti, & Shahid, 2015). The plant shows significant anticancerous activity in several cancer cell lines, however, the mechanism of signaling behind such activity has not been elucidated as yet.

11.4.53 *Ulmus wallichiana* (elm)

Cytotoxic and apoptotic effects on prostate cancer cells have been reported due to arresting of cells at the G0/G1 phase of the cell cycle by glucopyranosyl derivatives of the quercetin present in this plant (Kumar et al., 2015). The studies have been undertaken on the proapoptotic cascade-like increase of cytochrome c release, cleavage of caspase-3, and poly (ADP-ribose) polymerase in the treated cells.

11.4.54 *Verbena officinalis* (common verbena)

The report published by Encalada et al. (2015) has revealed that vicinal acetyl groups found in the anticancer phenylethanoid glycosides in the sugar rings extracted from this palnt show potential antiproliferative activity against colon cancer cell lines with IC_{50} values lower than 20 μg/mL. Chlorogenic acid in this species has potential in vitro antibacterial activity against *Escherichia coli*, *Proteus vulgaris*, and *Bacillus subtilis*. Aerial parts of this species contain polysaccharides which lead to a decrease in invasion, cell adhesion, and metastasis of colorectal cancer cells, as well as a decrease in the expressions of C-met, MMP-9, COX-2, and EP2, with an upregulation of E-CAD expression (Jin, Liu, Ma, Hua, & Jin, 2017).

11.4.55 *Viscum album* (mistletoe)

Lectins MLI-III identified in this plant are reported to be helpful in the inhibition of growth by causing apoptosis in colon cancers, gliomas, and sarcomas. Other compounds too are found in this species, such as betulinic, triterpenoic, oleanolic, and ursolic acids, which cause cell deaths in breast, gynecological, and lung cancers. A combination of all these leads to an activation of the caspase (CASP8 and CASP9) system causing apoptosis in Ewing sarcoma (Twardziok et al., 2016). The plant proves helpful in combination with many synthetic anticancer drugs. It inhibits growth in human breast carcinoma (HCC1937 and HCC1143), adenocarcinoma of pancreas (PA-TU-8902), prostate cancer (DU145), and lung carcinoma cell lines (NCI-H460). *V. album* extract used in the breast cancer cell line (HER2) along with synthetic drug "Trastuzumab" arrests cell cycle progression at both G0/G1 and G2/M stages by inhibiting the secretion of vascular endothelial growth factor (Weissenstein, Kunz, Urech, Regueiro, & Baumgartner, 2016). It acts as a potent immunostimulant via maturation and activation of dendritic cells which in turn help in imparting its anticancerous activity. Extracts of this plant also cause death of venous endothelial cells which are the main cells involved in tumorogenesis. ISCADOR grows on many other plants and shows a high rate of inhibition of NK cells and leads to growth inhibition in glioblastomas (Podlech, Harter, Mittelbronn, Pöschel, & Naumann, 2012). Increased immunostimulation can make this drug a potential cancer therapy and help to improve quality of life in chemotherapy patients.

11.4.56 *Zanthoxylum armatum* (winged prickly ash)

Z. armatum fruits and bark are rich in phenolics and flavonoids. These show antitumor, antioxidant, immunomodulatory, insecticidal, larvicidal, hepatoprotective, antimicrobial, and antiinflammatory features. According to Singh et al. (2015) leaf extract of this species induces DNA fragmentation, nuclear blebbing, and apoptosis via mitogen-activated protein kinases (MAPK) pathway by executing phosphorylation of extracellular signal-regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK) in the HeLa cells. Alam, us Saqib, and Waheed (2017) have reported that apoptotic

phenomena is observed in breast (MDA-MB-468 and MCF-7) and colorectal (Caco-2) cancer cell lines with nuclear fragmentation and chromatin condensation when treated with saponin-rich extract of this plant, which acts in a dose-dependent manner.

11.4.57 *Ziziphus mauritiana* (Indian jujube)

Cell cycle arrest, DNA fragmentation, and apoptosis in HL-60, Molt-4, HeLa cancer, and the normal cell line HGF are observed due to the involvement of ascorbic acid, thiamine, riboflavin-bioflavonoids, pectin A, betulinic acid colubrinic acid, alphitolic acid, oleanolic acid, betulonic acid, oleanonic acid, zizyberenalic acid and betulinic acid, jujubosides A, B, A1, B1, and C and acetyljujuboside B, saponin, and ziziphin present in the seed extract of this plant. Mishra, Khullar, and Bhatia (2011) have reported that in vivo studies on mouse models of Ehrlich ascites carcinoma show a decrease in tumor volume and viable tumor cell count with an increase in life span. The extracts of *Z. mauritiana* and *Punica granatum* applied in combination have produced a more pronounced effect and lead to apoptotic cascade activation of caspase-8 and -9 and p53 with the concomitant decrease in Bcl expression in Hela cell lines (Vakili and Parvizi, 2017). Betulinic acid isolated from this species is cytotoxic for brain tumor and neuroectodermal cells (Laszczyk, 2009; Pisha et al., 1995; Tan and Norhaizan, 2018; Zuco et al., 2002).

11.5 Important potential anticancer cultivated plants

11.5.1 *Abelmoschus esculentus* (okra)

Gastroprotective action is attributed to the lectin derivatives as they activate alpha-2 adrenergic and opioid receptors and decrease oxidation (Ribeiro et al., 2016). Monte et al. (2014) have reported that this species is a good source of antioxidative agents, particularly flavonoids/phenolics and its lectins significantly induce the apoptotic cell signaling pathway and growth inhibition up to 63% in MCF7 breast cancer cells, upregulation of proapoptotic signals like caspase-3, caspase-9, and p21 genes with a significant increase in the Bax/Bcl-2 ratio, all promoting selective antitumor activity in these cell lines. Its antiproliferative activity of has been found in colorectal adenocarcinoma, COLO-205 and retinoblastoma (Y79) (Gul et al., 2011). As such this plant proves to have potent anticancerous, antioxidant, antibacterial, and antiinflammatory characteristics, however, more studies are required to elucidate the signaling mechanisms in many other cancer cells.

11.5.2 *Allium sativum* (garlic)

The medicinal value of this species is mainly due to the presence of nearly 33 different organosulfur compounds found in this species (Bottone, Baek, Nixon, & Eling, 2002; Iciek, Kwiecień, & Włodek, 2009; Imadi et al., 2018). Chemopreventive effects of garlic extract and organosulfur compounds derived from this plant together with antioxidant features and tumor growth inhibition have been reported (Imadi et al., 2018; Thomson and Ali, 2003). These compounds inhibit cancer via detoxifying processes, arrest cell cycle, fuel the mitochondrial apoptotic pathway, and boost histones acetylation (Iciek et al., 2009; Imadi et al., 2018). Allyl sulfur compounds contribute a lot to anticancer activity by retarding chemically induced cancer at multiple sites in humans (Ariga and Seki, 2006; Bergès et al., 2004; Durak et al., 2007; Imadi et al., 2018; Milner, 2010; Seki et al., 2008; Song and Milner, 2001).

Garlic powder or garlic extract intake have been reported to reduce chemically induced skin and mammary gland carcinogenesis (Imadi et al., 2018). Amagase and Milner (1996), Sundaram and Milner (1996), Shirin et al. (2001), Sengupta and Das (2003), Sengupta, Ghosh, and Das (2004), Chung et al. (2004), Wu, Kassie, and Mersch-Sundermann (2005), and Matsuura et al. (2006) have reported that allyl sulfur compounds also protect colonic carcinogenesis via inhibition of carcinogen-induced DNA adduct formation, blockage of cell growth, cell proliferation, angiogenesis, induction of apoptosis, enhancement of carcinogen detoxifying enzymes suppression of carcinogen-activating enzymes, inhibition of cyclooxygenase-2 expression, scavenging carcinogen-induced free radicals, and inhibition of lipid peroxidation. They suppresses growth of human colon cancer cell lines, reduce the amount of cells in some phases, increase activity of caspase-3, and inhibit tubulin polymerization (Hosono et al., 2005; Imadi et al., 2018).

11.5.3 *Arachis hypogaea* (peanut)

Major constituents of peanut are resveratrol, piceatannol, eriodictyol, polyphenols, 5,7-dihydroxychromone, and luteolin, all with many health benefits (Ku, Chang, Cheng, & Lien, 2005). A high level of bioactivity in isolated phenols and

flavones is due to scavenging of free radicals and inducing antiproliferation in HeLa cancer cells with IC_{50} value of 34 μg/mL. A decrease in cell viability of HeLa cell lines has been reported for flavones particularly apigenin. Gaafar, Mahmoud, and Salama (2015) have reported cytotoxic effect on human cell line (HePG2−MCF7−HCT-116) following treatment with extracts of different parts of peanuts. Treatment with resveratrol rich extract of peanuts causes DNA double-strand break (DSB) and prolonged G2/M arrest occurs in radioresistant prostate cancer cell lines (Chen, Lien, et al., 2017). Other likely to be anticancer phytochemicals present in this plant are arachidin-1 and arachidin-3 (Shirley, 2017).

11.5.4 *Armoracia rusticana* (Japanese horseradish)

Significant antioxidative, anticancerous, and antiinflammatory characteristics are found in the highest concentration in Japanese horseradish due to the presence of allyl isothiocyanates (Sultana & Savage, 2008). 1,2-dilinolenoyl-3-galactosylglycerol and linolenoyloleoyl-3-β-galactosylglycero isolated from this plant induce dose-dependent cell cytotoxicity in lung, colon, and stomach cancer with an inhibition of COX inflammatory enzymes (Weil, Zhang, & Nair, 2005). High content of glucosinolates found in this species may also be responsible for antitumorogenic activity. According to Dekić et al. (2017) 5-phenylpentyl isothiocyanate (PhPeITC) a novel compound found in this plant induces cytotoxic effect in cancer cell line, Caco-2, HeLa, and noncancer MDCK cell lines.

11.5.5 *Avena sativa* (oats)

A. sativa plants contain phytochemicals like avenanthramides, an indole alkaloid−gramine, flavonoids, flavonolignans, triterpenoid saponins, sterols, tocols, and are rich in glucans, a polysaccharide with immense health-promoting effects. According to Daou and Zhang (2012) the latter are anticancerous and mediate with the immune responses by activating macrophages and increasing levels of immunoglobulin, NK cells, and killer T-cells, which can help in combating cancers of various types. Gamma-irradiated oat β-D-glucan causes cytotoxic effects against colo-205 and MCF7 cancer cell lines but no effect is seen in the normal cell (Shah et al., 2015). Choroma-Ska, Kulbacka, Harasym, Dubi-Ska-Magiera, and Saczko (2017) have reported that a combined effect of electroporation and oat β-D-glucan produces a stronger cytotoxic effect on human melanoma cells (Me45) with a marked increase in the expression of cytochrome c which helps in the initiation of caspases. They have also observed that high-molecular-weight oat β-D-glucan compared to low-molecular-weight ones cause cytotoxicity in human epithelial lung cancer by inducing lipid peroxidation and increase superoxide dismutase (SOD) expression. Oat-derived β-D-glucan leads to a concentration-dependent increase of apoptotic molecules like caspase-3/7 followed by arrest of cell cycle in G1 phase in human skin melanoma, HTB-140 cells lines (Parzonko, Makarewicz-Wujec, Jaszewska, Harasym, & Kozłowska-Wojciechowska, 2015).

11.5.6 *Brassica juncea* (mustard)

Mustard leaf extracts show antioxidant, antiinflammatory, antiischemic, and antideoressant activities. Kim, Kim, and Park (2007) have shown that methanolic extract of mustard leaf has antimutagenic and antitumorogenic effects in gastric adenocarcinoma cells (AGS) and colon carcinoma cells (HT-29) in humans; fermented leaf kimchi is responsible for more inhibition of cancer cells. Ethanolic extract of mustard leaf shows anticancer activity in HCT-116 colorectal cancer cells and H1299 nonsmall cell lung carcinoma cells in vitro. Kwak, Lee, and Ju (2016) have shown that the architecture of the nucleus changes and angiogenesis decreases, together with metastasis and arrest in the G1 phase of the cell cycle. Studies on the cancer cell lines have revealed that proangiogenic factors like vascular endothelial cell growth factor and basic fibroblast growth factor are significantly reduced. Allyl isothiocyanate from *B. juncea* causes growth inhibition of cancer in in vivo as well as in vitro models. According to Bhattacharya et al. (2010) its seed powder is rich in isothiocyanates and causes apoptosis by modulating vascular endothelial growth factor, cyclin B1, caspase-3, and G_2/M phase arrest in bladder cancer cell lines in vitro.

11.5.7 *Brassica napus* (rapeseed)

The rapeseed is rich in beneficial health compounds which include ascorbic acid, minerals, phenolics, and glucosinolates (GLS), all acting as potent antioxidants and anticancer agents. Significant anticancer activities due to peptides derived from rapeseed hydrolysate have been reported. The extract shows cytotoxic effect with DNA damage, cell cycle arrest in S phase, and drastic morphological changes in HeLa cell lines (Xue, Liu, Wu, Zhuang, & Yu, 2010). Pectins

present in the rapeseed cakes too are promising anticancerous agents. Greater growth inhibition in MCF-7 cells than Caco-2 has been recorded when treated with pectin-rich rapeseed extract (Cobs-Rosas, Concha-Olmos, Weinstein-Oppenheimer, & Zuniga-Hansen, 2015). Rapeseed peptide isolates have shown a dose-dependent anticancerous activity by inducing apoptosis in the cervical cancer cell lines and arresting cell cycle (Xue, Yu, Liu, Wu, & Wang, 2011).

11.5.8 *Brassica oleracea* (cabbage)

B. oleracea is rich in phenolic compounds which show anticancer, antioxidant and antiinflammatory activities. Several nonpolar extracts of this species show activities against colon adenocarcinoma, breast, lung, and kidney carcinomas, but activity is more prominent with selenium-fortified extract against all these cancers (Bachiega et al., 2016). According to Ye et al. (2011) some antifungal proteins from red cabbage decrease nasopharyngeal cancer cells and hepatoma cell proliferation with prominent antioxidant activities. In vitro cytotoxicity has been reported on treating HeLa and PC-3 human cancer cell lines with a 2-pyrrolidinone-rich fraction of cabbage with an arrest of cell cycle in G0/G1 phase (Thangam et al., 2013). Devi and Thangam (2012) have reported that a prominent isothiocyanate—sulforaphane—found in this plant shows antiproliferative effects in the human epithelial carcinoma, HEp-2 cell line, via apoptotic mechanism by decreasing expression of antiapoptotic protein, bcl-2, while upregulating proapoptotic proteins Bax and caspase-3. This compound inhibits the proliferation of prostate cancer cell line by induction of apoptosis.

Similarly Hafidh et al. (2013) have reported that in the culture of HeLa and HepG2 cancer cell lines, there is an increase in anticancer cytokines, TNFα and IFNβ, with an arrest of cell cycle in G0 phase and activation of apoptotic cascade proteins. Anticancer activity for different types has been recorded for hydrolysis products of glucosinolates, but a low effect has been seen on prostatic, endometrial, and ovarian cancers (Imadi et al., 2018).

11.5.9 *Brassica rapa* ssp. *rapa* (turnip)

Several medicinally important phytocompounds like terpenoids (limonene), vitamin C, tangeretin, nobeltin, limonoid, and flavonoids with antioxidant and anticancerous features are found in turnip. Kausar and Waris (2017) have reported that turnips are important antioxidative agents and can be used to cure cancers indirectly by increasing the antioxidant profile of the body. Its glucosinolates show antimicrobial and anticancerous activity and can be used as antibacterials against *Vibrio parahemolyticus*, *Staphylococcus aureus*, and *Bacillus cereus*. β-Phenylethyl isothiocyanate is the abundant glucosinolate derivative present in this plant and inhibits the growth of hepatoma cell line (HepG2) in a dose-dependent way with IC50 of 24.5 μM (Hong and Kim, 2008).

11.5.10 *Camellia sinensis* (green tea)

Epigallocatechin-3-gallate (EGCG) is the active constituent of green tea (Siddiqui, Hareramdas, Abbas, Parween, & Khan, 2018). Many of the cancer formations and developments are inhibited by its polyphenols as studied in the animal models (Imadi et al., 2018; Yang & Wang, 2010). Aucamp, Gaspar, Hara, and Apostolides (1997) and Cooper, Morre, and Morre (2005) have reported that catechins present in *C. sinensis* extracts as antioxidants inhibit the production of ROS, thus reducing the cancer. It also inhibits topoisomerase II enzyme cytotoxicity to hepatoma cell line, thus resulting in its chemopreventive activity (Ramirez-Mares, Chandra, & De Mejia, 2004).

Ravindranath, Ramasamay, Moon, Ruiz, and Muthugounder (2009) have stated that different melanoma-metastasized cell lines are inhibited by EGCG; catechins also inhibit pancreatic ductal adenocarcinoma, and regular intake inhibits carcinogens present in tobacco, reducing lung cancer risk in smokers (Imadi et al., 2018; Kurbitz et al., 2011; Liang, Binns, Jian, & Lee, 2007).

11.5.11 *Capsicum annum* (pepper)

Important bioactive molecules present in the *Capsicum* are quercetin, luteolin, catechin, epicatechin, kaempferol, myricetin, apigenin, rutin, and capsaicinoids. According to Mori et al. (2006) treatment with capsaicin shows that p53, p21, and Bax gene expression is upregulated in various prostate cancer cell lines (LNCaP, PC-3, and DU145), leading to a decrease in cell growth and proliferation of cancer cells. This compound also activates various kinases involved in antiproliferative signaling cascades like MAPkinase, ERK, and JNK (Kang et al., 2003). Epigenin derived from capsicum is known to induce growth inhibition in breast cancers via activation of different kinases (Miean & Mohamed, 2001).

Oral capsaicin controls human pancreatic cancer (Zhang, Humphreys, Sahu, Shi, & Srivastava, 2008). Lee, Richardson, Dashwood, and Baek (2012) have observed that it has a preventive role in human colorectal cancers by suppressing different mechanisms.

11.5.12 *Crocus sativus* (saffron)

C. sativus is a well-known medicinal plant, with mainly stigmas, as well as petals, leaves, and stems evaluated for medicinal purposes (Kafi, Kamili, Husaini, Ozturk, & Altay, 2018; Srivastava, Ahmed, Dixit, & Dharamveer, 2010). The name saffron is mainly used for the dried, dark red stigmas of this species. It has been studied using animal models and has demonstrated antitumor and cancer-preventive activities with potent anticancer and antitumor features (Abdullaev, 2002; Imadi et al., 2018).

11.5.13 *Cucurbita pepo* (pumpkin)

The pumpkin rind, seed, flesh, and defatted seed meal extracts are rich in essential nutrients like fatty acids (oleic, linoleic, palmitic, and stearic), tocopherols, and sterols, all being helpful as antioxidants, anticancerous, antidiabetic, and antibacterial agents. *C. pepo* inhibits lipid peroxidation, helping in the repair of DNA mutations as well as other chromosomal abnormalities leading to its antimutagenic effect. The oil from this species decreases IPSS (International Prostate Symptom Scores) by 41.4%, it can therefore be used as a cure against benign prostratic hyperplasia. According to Zaineddin et al. (2012), consuming a mixture of pumpkin seeds with soybeans and sunflower can be useful for lowering risk of postmenopausal breast cancers. The findings published by Shaban and Sahu (2017) have revealed that "Moschatin" isolated and characterized from *Cucurbita moschata* mature seeds—a novel imuunotoxin—significantly decreases the growth of melanoma cell lines, M21. Asif et al. (2017) also have studied the peel and pulp puree of pumpkin and reported that it has an antiproliferative effect in MDBK cancer cell line.

11.5.14 *Daucus carota* ssp. *sativus* (black carrot)

This taxon is rich in anthocyanins acting as natural coloring agents, these are safe to use. Netzel et al. (2007) have reported that anthocyanins extracted from this plant have shown a dose-dependent antiproliferative activity in human colorectal carcinoma (HT-29) and leukemia cell lines (HL-60). Ethanolic extract of *D. carota* ssp. *sativus* used for the treatment has shown a significant cytotoxic effect in rat neuroblastoma cell lines (Neuro 2A) (Sevimli-Gur, Cetin, Akay, Gulce-Iz, & Yesil-Celiktas, 2013); but the same extract has caused less cytotoxicity in normal cell line VERO (African green monkey kidney) which showed a significantly high IC_{50} of 170.13 μg/mL. In view of this, carrot extracts can significantly inhibit cancer cell proliferation with no cytotoxicity in the normal cells of the body. Yesil-Celiktas, Pala, Cetin-Uyanikgil, and Sevimli-Gur (2017) have reported that anthocyanins from this plant—used in silica-PAMAM dendrimer nanoparticles—have shown that they lead to a significant cytotoxicity in neuroblastoma cell lines. According to Shebaby et al. (2013) oil extract (DCOE) from *D. carota* in general has remarkable antioxidative characteristics depicting a dose- and time-dependent decrease in cell proliferation in human colon cancer (HT-29, Caco-2) and breast carcinoma cell lines (MCF-7, MDA-MB-231).

11.5.15 *Foeniculum vulgare* (fennel)

Fennel contains a phenylpropene derivative known as anethole showing antimicrobial, insecticidal, estrogenic and galactogogue activity and shows potent anticancerous characteristics. This compound is also cytotoxic showing effects similar to cyclophosphamide, with potent antimitotic activity. The findings reported by Al-Harbi et al. (1995) have shown that it increases survival rate, reduces tumor weight, and decreases MDA in Ehrlich ascites carcinoma. There has been a mediation in the antiinflammatory response in DMBA induced in a rat mammary cancer model and also inhibition of H_2O_2, phorbol myristate acetate, or TNF alpha following application of anethole and its derivative activated NF-κB (Lubet et al., 1997). According to Nakagawa and Suzuki (2003) estrogenic effect in MCF-7 in mammary cells and considerable cytotoxicity in rat hepatocytes has been recorded due to biotransformation of anethole to transanethole. If anethole dithiolethione, an organosulfur compound, is given to smokers with bronchial dysplasia in a clinical trial chemoprevention occurs (Lam et al., 2002). No study has been undertaken on the anticancer activity of pure extract of fennel but its phytoconstituents and their derivatives show potent activity against cancer.

11.5.16 *Fragaria ananassa* (strawberry)

Several biological characteristics, such as acting as antioxidant, anticancer, antineurodegerative, and antiinflammatory agents, have been reported for strawberries which is associated to the many phytochemicals they contain, for example, vitamin C, hydroxycinnamic acids, anthocyanins, and flavonoids. A high concentration of elagic acid has been reported from this plant which has potent anticarcinogenic and antimutagenic activity. According to Seeram et al. (2006) its extracts are seen to produce the highest inhibition of growth and apoptotic activity among all berries against human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT-116), and prostate (LNCaP) tumor cell lines at concentrations varying in different cell lines. Treatment with ethanolic extracts of four strawberry varieties has revealed that growth of cervical cancer cell line, CaSki and SiHa lines, and breast cancer cell lines, MCF-7 and T47-D, is inhibited (Wedge et al., 2001). Somasagara et al. (2012) have reported that the extracts activate several intrinsic pathways for apoptosis, particularly in breast cancer cells. They further suggest that increased expression of p73, a p53 analogue, and overexpression of BAX, caspase-9, and cytochrome C is found in T47D breast cancer cell lines when treated with methanolic extract of strawberry fruits. There are changes in oral epithelial gene expression and intraoral metabolites in a manner favoring anticarcinogenic profile, this being observed when smokers consuming tobacco are given strawberries orally, paving a novel way to curb the health disadvantages faced by smokers (Ahn-Jarvis et al., 2017).

11.5.17 *Glycine max* (soyabean)

Soy products like raw soyabean, soy milk, tofu, and others have a number of health benefits. Their antioxidant properties are due to the presence of saponin, flavones, and phytates. The isoflavones in soyabean are daidzein, genistein, and its β-glucoside conjugate. All these are reported to decrease proliferation of cancer cells because of their important antiangiogenesis activity. Anticancer activity in MCF-7 breast cancer cell lines has been recorded after using soy products. It is directly related to the genistin levels (Somdee, Mahaweerawat, Wibulutai, Dungkokruad, & Yungyuen, 2017). Khan and Kang (2017) have reported that an upregulation of Bax and downregulation of BCl2 apoptotic proteins through JNK signaling pathway has been observed after using fermented soybean extract leading to a dose-dependent reduction in growth in HCT-116 colon cancer cell lines. Cell adhesion and metastasis result from matrix metalloproteinases (MMPs); the MMP inhibitors in soybean significantly decrease growth of colorectal cancers and antiinflammation in inflammatory bowel disease and colitis as reported by Lima, Oliveira, Mota, and Ferreira (2017). Soybeans also contain β-conglycinin and glycinin peptides rich in glutamine which inhibit proliferation in Caco-2, HT-29, and HCT-116 cell lines (González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018). Some of the soybean peptides are gastrointestine-friendly and inhibit the proliferation rate of blood (CCRFCEM and Kasumi-3), prostate (PC-3), and breast (MCF-7) cancer cell lines; these can be used as food supplements to the cancer patients, as proposed by Rayaprolu et al. (2017). The data published by Soni, Femida, and Sharma (2017) show that soybean saponins are also responsible for antioxidant and cytotoxic effects in breast cancer cell-line.

11.5.18 *Helianthus annuus* (sunflower)

Some of the essential components in sunflower oil are ferulic, caffeic, chlorogenic acids, and *p*-coumarin, with all having high antioxidative activities. Other phytochemicals found in this plant are Heliespirone, Heliannuol E, and Helikauranoside A. According to Al-Jumaily, Al-Shamma, Al-Halbosiy, and Al-Shamma (2013) a significant reduction in the tumor size has been observed when sunflower seed oil (SSO) is injected subcutaneously. The sunflower trypsin inhibitor (SFT1) is a protein which is reported to inhibit enzymes specific for breast cancer. Growth inhibition in many cell lines including HeLa, rhabdomysarcoma (RD), and gliomas have been recorded by Al-Jumaily et al. (2013) after using extracts from sunflower seeds. Pinedo et al. (2017) have stressed the fact that sunflower lectin (Helja) expressed in *E. coli* causes cell toxicity to human neuroblastomas. Sunflower seeds are also rich in antioxidative enzymes like SOD, POD, GPX, GR, and CAT, all present in high concentrations, and these in turn help in treating cancers.

11.5.19 *Helianthus tuberosus* (artichoke)

The dried artichoke is reported to be rich in proteins, crude fiber, minerals, starch, pectin substances, and inulin. Antioxidative, antiinflammatory, and anticancerous activities of this plant are due to the high content of total phenolics. According to Zhang and Kim (2015) a dose-dependent cytotoxicity in human lung epithelial A549 cells lines is induced by different extracts of artichoke with the highest effects by water extract, but the exact mechanism behind this

inhibition needs further evaluation. Potential cytotoxic activity against 1031 leukemia and HCT-116 cell lines has been seen after using artichoke leaf-derived sesquiterpene lactones; but heliangine has shown moderate activity against Hep G2 and breast cancer MCF7. However, inulins isolated from artichoke have not prevented carcinogenesis (Baker, El Gengaihi, Enein, & El Ella, 2010). Polysaccharide-rich fraction of artichoke has inhibited the Hep-2 (laryngeal) and L-929 (mice fibroblasts) cell lines in a dose-dependent manner as a possible method of immunomodulation due to the increase in level of NK-cells as reported by Evgenii (2015). It decreases metastasis in these cancers. 3-Hydroxy-8β-tigloyloxy-1,10-dehydroariglovin, a novel sesquiterpene lactone from artichoke, and 10 other sesquiterpene lactones have shown prominent antitumorogenicity against MCF-7, A549, and HeLa cancer cells lines, whereas flavones have shown selective inhibitory activity only against HeLa cell lines (Yuan et al., 2013).

11.5.20 *Hordeum vulgare* (barley)

β-Glucan fiber, phenolic acids, flavonoids, lignans, tocols, phytosterols, and folate are the constituents in barley, all possessing many health benefits as they act as antioxidants and are antiproliferative and antilipidemic (Idehen, Tang, & Sang, 2017). A decrease in the cell viability of human colon adenocarcinoma (HT-29) and human lung adenocarcinoma (A549) cell lines has been observed after applying young barley extracts but less cytotoxicity is seen in normal cell lines, colon epithelial cells (CCD 841 CoTr), and human skin fibroblasts (HSF) (Czerwonka, Kawka, Cykier, Lemieszek, & Rzeski, 2017). Antiproliferative action in nude mice transplanted subcutaneously with human HT-29 cells has been seen after applying *Lactobacillus plantarum* fermented barley, causing transcriptional regulation of Bax, Bcl-2, caspase-3, and cyclin D1 genes and inducing cellular apoptosis as reported by Fang, Zhang, Xiang, Ying, and Zhou (2017). The 3,4-dihydroxybenzaldehyde derivative of barley is reported to block H_2O_2-induced tumor development by protecting free radical-induced DNA damage (Jeong, Hong, & Jeong, 2009).

11.5.21 *Ipomoea batatas* (sweet potato)

The polyphenols and flavonoids present in this plant give it high degree of free radical scavenging characteristic, even the polysaccharides extracted from it show antioxidant activity and effective inhibition of tumor growth in rats (Yuan et al., 2017). The immune factors IL-2, TNF-α, and VEGF of sweet potato show an immunomodulatory effect on SPG-56 glycoprotein isolated from this species which suppresses colon cancer growth in both in vivo and in vitro conditions, as reported by Wang et al. (2017). According to Ogutu, Mu, Sun, and Zhang (2018) significant antiproliferation in HT-29 colon cancer cells is observed due to the pectin-rich fraction of *I. batatas* by inducing caspase-3-mediated apoptosis and cell death. The data published by Zhang and Mu (2018) depict that the alcalase enzyme-treated protein fraction of sweet potato is highly antiproliferative by causing G2/M cell cycle arrest, increasing p21, Bax, and caspase-3 expression, and decreasing Bcl-2 expression in HT-29 colon cancer cell lines. Leaves of this plant are rich in polyphenols; its leaf phenol-rich fraction shows significant antiproliferative activity in many prostrate cancer cell lines without side effects in normal prostate epithelial cells (Karna et al., 2011). Activation of caspases and pronounced DNA degeneration is responsible for antiproliferative activity of sweet potato leaves.

11.5.22 *Lagenaria siceraria* (calabash or white-flowered gourd)

In albino rats induced skin papillomagenesis is significantly decreased by including in their diet bottle gourd juice which contains 7,12-dimethylbenz(a)anthracene (DMBA) (Kumar, Kale, & Tiku, 2013). A revival of cellular architecture and reduction of dermal infiltration is seen in tissues of papilloma. Treatment with methanolic extract of leaf of *L. siceraria* in rats induced with Ehrlich ascites carcinoma has shown a significant decrease in growth (Saha, Sen, Bala, Mazumder, & Haldar, 2011). Triterpenes present in this plant, such as friedooleanane, cause cytotoxic activity against the SK-Hep 1 cell line significantly (Chen, Chen, & Chang, 2008).

11.5.23 *Linum usitatissimum* (linseed)

The plant is rich in some biologically active important compounds like omega-3, omega-6 fatty acids, linolenic acid, high-quality proteins, and fibers; it also has high amounts of other phytochemicals, like phenolic acids, cinnamic acids, flavonoids, and lignans. The main lignan found here is secoisolariciresinol diglucoside, which shows potent antioxidant features. Danbara et al. (2005) and Marghescu, Teodorescu, and Radu (2012) report that extracts from this species inhibit breast and colon tumors significantly by inducing apoptosis and cell death. Significant decrease has been

recorded in the severity of late-stage ovarian tumors in hens fed with a 10% linseed-enriched diet (Ansenberger et al., 2010). Podophyllotoxin extract from linseed encapsulated in gold nanoparticles has anticancer effects in many cancer cell lines, such as colorectal (HT-29), lung (A549), and breast (MDA-MB-231) cancer cell lines, as reported by Safarpoor et al. (2017). Nanoemulsion of sorafenib with flaxseed oil increases the antitumor activity of sorafenib by decreasing tumor volume along with reducing hepatotoxicity of the sorafenib drug (Alkhatib, Nori, & Al-Ghamdi, 2017). Flax oil generated from transgenic flax seeds leads to a significant reduction in cell survivability of mammalian cancer cells and also increases wound healing by increasing skin fibroblast proliferation as reported by Barowski, Bczak, Wiatrak, and Kulma (2017).

11.5.24 *Malus domestica* (apple)

The peels of *M. domestica* are good source of triterpenoids like ursolic acid. Latter shows antiinflammatory, antioxidative, and antiproliferative properties. The extract from cultivar "Gala" decreases proliferation of breast cancer, Mcf-7and Mcf-7: Her18 cells and prostate cancer, CWR22Rν1 and DU145 cell lines (Reagan-Shaw, Eggert, Mukhtar, & Ahmad, 2010). These cells also show a decrease and increase in the levels of cancer specific proteins PCNA (proliferative cell nuclear antigen) and maspin (a tumor suppressor protein). A flavonoid enriched fraction of its peels inhibits growth in human hepatocellular/liver cancer cell, HepG2 (Sudan & Rupasinghe, 2014). Cancer cell death is due to activation of caspase-3, arrest in cell cycle at G2/M phase and inactivation of topoisomerase leading to aberrations in DNA, leading to apoptosis. Whole apple fruit freeze dried powders have shown a significant growth inhibition in the human breast cancer cell line, MDA-MB-468 induced with nitrosaurea carcinogenesis (Thompson, Stushnoff, McGinley, & Thompson, 2009). According to McCann et al. (2007) phenolics present in this fruit show antioxidative characteristics, can alleviate the DNA damage, decrease progression of stages of cell cycle and inhibit cell invasions in colorectal cancers.

11.5.25 *Nigella sativa* (black cumin)

N. sativa with its effective antioxidant activity is publically known as heavenly gift in sacred books and has been used as an anticancer drug since ages. Several nutritionally important fatty acids, volatile oils (nigellone, thermoquinone, thermohydroquinone), alkaloids (nigellicine, nigellidine), coumarins, saponins and many minerals are found in this plant. A potential anticancer agent alpha-hederin has been reported from its extract, which has proved effective in lung carcinoma cell (A549) and colon carcinoma cell (DLD-1) inhibition when a hexane extract of its seeds is used, showing antiinflammatory action in these cells as reported by Bourgou, Pichette, Marzouk, and Legault (2012). In vivo study using oils extracted from the seeds of this species has revealed a decrease in the growth of solid tumor in the mouse model. Ait Mbarek et al. (2007) have studies the antitumorogenic activity of its different extracts and they mention that the action varies in different cell lines, in particular when tested in murine mastocytoma cell line (P815), monkey (Vero) and hamster (BSR) kidney carcinoma cell lines and sheep heart carcinoma (ICO1) cells. According to Salomi, Nair, Jayawardhanan, Varghese, and Panikkar (1992) fatty acid fraction of *N. sativa* seeds too show antitumor activity against many carcinoma types such as; Ehrlich ascites carcinoma (EAC), Dalton's lymphonia ascites (DLA) and Sarcoma-180 (S-180) cells by a certain mutation in DNA molecule. In the lymphoma cells (U937) proapoptotic molecules like caspase-3, BAD, and p53 gene expressions have increased with a significant increase in DNA fragmentation as reported by Arslan, Isik, Gur, Ozen, and Catal (2017). A suppression of tumor growth is seen due to p53 as it activates downstream signaling molecules like caspase-3 and BAD. Thymoquinone; an essential oil; shows wide range of activities as antiinflammatory and antineoplastic in many cancers. A significant decrease in growth is observed in the head and neck squamous cell carcinoma (HNSCC) cell lines after thymoquinone is given alone or in combination with radiation therapy (Kotowski et al., 2017). A growth inhibition has also been recorded in the case of MDA-MB-231 breast cancer cell line following treatment with ethanolic extract of seeds of this species (Banerjee et al., 2017). Thymoquinone seems to help in the tumor regression by effecting antiproliferative molecular machinery like p53, p73, phosphatase and tensin homolog, peroxisome proliferator-activated receptor-γ and the activation of caspases.

11.5.26 *Ocimum basilicum* (basil)

Two essential phytochemicals found in *O. basilicum* are the rosmarinic and caffeic acid, which show several medicinally important characteristics like antibacterial, antiinflammatory, antiproliferative/anticancer, antioxidant, antiviral and antifungal activities. Its pharmacologically essential properties are carminative, antispasmodic, diuretic, antiseptic,

anesthetic, analgesic and antitussive. An increase in the antioxidant and antiproliferative activity against a breast cancer cell line (MCF-7) has been reported after applying UV-B irradiated leaves following its harvesting (Ghasemzadeh et al., 2016). According to Kusamran, Ratanavila, and Tepsuwan (1998) the leaves of this species effectively prevent chemical carcinogenesis in different organs of rats by increasing glutathione S-transferase activity. Its essential oils contain eugenol, isoeugenol and linalool which show an important cytotoxic activity particularly against SKOV3 ovarian cancer cell lines (Zarlaha, Kourkoumelis, Stanojkovic, & Kovala-Demertzi, 2014). The reason for the antiproliferative activity is probably due to downstream inhibition of cyclooxygenase and lipoxygenase enzymatic action. Betulinic acid a triterpenoid derivative from this plant has unique anticancerous action against melanoma cells without damaging normal cell lines as reported by Pandey, Pandey, Singh, Gupta, and Banerjee (2015). A significant elevation of bioavailability of polyphenols and rosmarinic acid follows after elicitation of basil with arachidonic acid which increases its antioxidant and anticancerous activity (Złotek, Szychowski, & Świeca, 2017). Higher anticarcinogenic and cytotoxic activity in MCF-7 cell is seen after applying essential oil from basil show as compared to its methanolic extracts but, the mechanism for cytotoxicity is still unknown (Mohammadi, Majd, Nejadsattari, & Hashemi, 2014). According to Kathirvel and Ravi (2012) methyl thiazol tetrazolium rich fraction in the basil oil shows potential anticancer activity by causing cytotoxicity against the human cervical cancer cell line (HeLa), human laryngeal epithelial carcinoma cell line (HEp-2) and NIH 3T3 mouse embryonic fibroblasts with IC_{50} values of 90.5−96.3 μg/mL.

11.5.27 *Oryza sativa* (rice)

O. sativa is rich in neutraceuticals with great health benefits. γ-Oryzanol, ferulic acid, caffeic acid, tricin, and β-sitosterol are the bioactive molecule found in rice which are responsible for a decrease in proliferation of many cancer cell types. Das, Patra, Choi, and Baek (2017) have reported that cancer growth and development in humans is inhibited by brown basmati rice consumption. These workers further state that rice contains tocotrienols and polyphenols which are responsible for anticancer activities in various types of cancers. According to Hui et al. (2010) black rice extract is rich in anthocyanin and its extract is responsible for apoptosis by activating caspases, disrupting mitochondrial membrane potential, cleaving poly (ADP-ribose) polymerase (PARP) and suppressing the expression of angiogenesis proteins, MMP-9, MMP-2, and uPA in breast cancer cell lines, MCF-7, MDA-MB-231, and MDA-MB-453. Rice fermented with *Lentinus edodes* is also reported to show dose-dependent inhibition in Sarcoma-180 cells by activating natural killer cells and increasing immune responses as mentioned by Kim, Kim, Yang, et al. (2007). Rice bran is rich in glycoproteins which causes immunomodulation and a prominent decrease in metastasis of colon carcinoma cells with a concomitant increase in secretion of cytokines like tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12 (Park, Yoon, Lee, Kim, & Choi, 2017).

11.5.28 *Panicum miliaceum* (finger millet)

Seed coat of *P. miliaceum* is a rich source of phenolic compounds (mostly derivatives of benzoic acid). These are reported to show potent antioxidant activity. A predominant phenol in this species is proto-catechuic acid. In rats an increase in the levels of catalase, glutathione peroxidase, and glutathione reductase is seen following feeding with diet rich millet which according to Hegde, Rajasekaran, and Chandra (2005) lead to anticarcinogenic activity. Cytotoxicity in adipocytes is observed by loss of mitochondrial membrane potential, activating caspase-3 and PARP degradation due to butanolic fraction of millets (Lee et al., 2014). Zaki et al. (2017) have studied pennogenin, yamogenin, yamogenin and pennogenin glucopyranosides; the four steroidal saponins; isolated from millets, all showing cytotoxic activities against many mammalian cancer cell lines

11.5.29 *Phaseolus vulgaris* (bean)

P. vulgaris shows antioxidant, antiinflammatory and anticancerous features because of high phenolics, saponins and flavonol contents. The results published by Aparicio-Fernández et al. (2006) on methanolic extract of its seed coats reveals that toyopearl and silica gel fractions show significant anticancerous activity against human adenocarcinoma cells (HeLa); but lesser effect has been observed in human premalignant keratinocytes (HaCaT). Extracts of germinated its seed coats also cause cytotoxicity in many cancer cell lines with no effect on normal cells. Genistein and flavonols found in the beans significantly inhibit the growth of mammary and hepatic and colon cancer cells (Guajardo-Flores, Serna-Saldívar, & Gutiérrez-Uribe, 2013). Azoxymethane induced colon cancer in rats is ameliorated by polysaccharide rich fraction of this plant by inducing apoptotic gene cascade (Feregrino-Pérez et al., 2008). Beans also show an

antiinflammatory effect which helps much in its antiproliferative action. According to Heredia-Rodríguez, de la Garza, Garza-Juarez, and Vazquez-Rodriguez (2017) managing of inflammation in lipopolysaccharide-induced macrophages is due to downregulation of cyclooxygenase-2 expression, prostaglandin E2 production, NF-κB pathways and nitric oxide production which in turn are responsible for the anticancerous activity of beans. Li, Liu, et al. (2017) have reported that trypsin inhibitor peptide of common beans inhibits [methyl-^{3}H] thymidine incorporation in leukemia L1210 cells and lymphoma MBL2 cells, and can be used as a potent agent for ameliorating cancers.

11.5.30 *Phyllanthus emblica* (amla)

Phytochemical analysis data published by Baliga and Dsouza (2011) and Baliga et al. (2018) reveal that *P. emblica* is rich in vitamin A together with gallic, ellagic, chebulinic, citric and chebulagic acids, as well as several other active compounds. Its cytotoxic activity has been investigated using different cell lines as reported by Jose, Kuttan, and Kuttan (2001). It induces apoptosis in Dalton's lymphoma ascites and CeHa cell lines (Rajeshkumar, Pillai, & Kuttan, 2003). However, in Chinese hamster ovary cell lines no cytotoxicity has been recorded, suggesting that it shows selective cytotoxic activity against neoplastic cells (Sumantran et al., 2007). In the case of Dalton's lymphoma administration of amla has effectively reduced growth of solid tumor and increased the survival time (Baliga et al., 2018; Jose et al., 2001). Whole extracts as well as amla phytochemicals have shown antiproliferative effects as mentioned by Zhang et al. (2004).

Past studies have also confirmed that amla possesses chemopreventive action, being useful in inhibiting chemical-induced skin, hepato as well as oral carcinogenesis (Baliga et al., 2018; Rajeshkumar et al., 2003; Sancheti, Jindal, Kumari, & Goyal, 2005). All these reports show that amla is a useful antimutagenic and anticarcinogenic agent and validates its regular use for health benefits (Baliga et al., 2018).

11.5.31 *Piper betle* (betel vine)

Kudva et al. (2018) mention that leaf shows antimicrobial, anticariogenic, and several other effects as per the preclinical experiments. However, as against all these beneficial effects, the leaves are cited as a cause of cancer, mainly because they are a part of betel quid containing tobacco and areca nut-both known as proved carcinogens (Kudva et al., 2018). A common belief is that regular consumption of betel leaf causes oral cancer, reason being that the leaves are generally chewed in the form of 'betel quid' which includes *Areca catechu*, betel leaf, and slaked lime. *Nicotiana tabacum* is also added to the mixture which is a known culprit of oral cancer (Kudva et al., 2018). Many investigations have been undertaken with betel quid constituents; results have conclusively revealed the fact that tobacco as well as areca nut are carcinogenic and slaked lime promotes carcinogenesis (Kudva et al., 2018). Other scientifically proved fact is that the leaf is devoid of mutagenic and carcinogenic effects, because Bhide, Shivapurkar, Gothoskar, and Ranadive (1979) have shown for the first time that, aqueous extract of its leaves fails to cause tumors in both Swiss and C17 mice, proving it is not carcinogenic.

A considerable *Helicobacter pylori* infection and even higher gastric cancer incidences are reported by Jemal et al. (2011), Torre et al. (2015), and Kudva et al. (2018) in Asian countries with considerable consumption of betel quid. Supplementation of leaf extract with drinking water has significantly reduced forestomach neoplasia (Bhide, Zariwala, Amonkar, & Azuine, 1991). According to their findings anticarcinogenic activity is concentration dependent. Studies with betel phytochemicals have revealed an equally effective prevention against forestomach tumorigenesis in mice. The phytochemicals in the betel leaves in general prove protective against the gastric cancer (Kudva et al., 2018).

11.5.32 *Piper nigrum* (black pepper)

Black pepper is mainly consumed as a spice but also used in folk medicine. Srinivasan (2007) and Siddiqui et al. (2018) have given detailed information on its much studied active constituent the piperine. Their anticancer activity has been observed in lung metastasis piperine study (Pradeep & Kuttan, 2002). This compound is inhibitory in mammosphere formation (Kakarala et al., 2010) and its inhibitory effect is in the metabolic activity of human rectal adenocarcinoma cells via prevention of cell cycle progression and induction of apoptosis (Yaffe, Power Coombs, Doucette, Walsh, & Hoskin, 2012).

11.5.33 *Pisum sativum* (pea)

This species has protease inhibitors like rTl1B and rTl2B which cause growth inhibition in colorectal cancer cell lines, HT-29 (Clemente, Gee, Johnson, MacKenzie, & Domoney, 2005). Lectins present in the leaves show growth reduction in breast, liver, larynx, and colon cancer cell lines (El-Aassar, Hafez, El-Deeb, & Fouda, 2014; Patel, 2014). According to Stanisavljević et al. (2016) seeds too are a rich source of several phenolics with chemopreventive action and growth inhibition in many cancer cell lines like lung (A594), breast (MDA-MB-453), colon (LS174), and blood (K562).

11.5.34 *Prunus dulcis* (almond)

P. dulcis is rich in vitamins particularly vitamin E which plays a big role in maintaining hair, skin and in general the cellular membranes. Main compounds present in the almond are amygdalin and prunasin. These show antioxidant, anti-inflammatory, antibacterial and anticancer activities. Dose-dependent arrest of bladder cancer cells in G0/G1 stage has been ascribed by Makarević et al. (2014) to amygdalin. According to Lee and Moon (2016) it inhibits proliferation of MCF7, MDA-MB-231 and Hs578T breast cancer cell lines by regulating the downstream apoptotic genes and decreasing adhesion of Hs578T cell line. In lung, hepatic (Hep-G2) and colon (HCT-116) cancer cell lines antimetastatic and cytotoxic effects have been recorded. In human prostate cancer, DU145 and LNCaP cells it induces apoptosis by activating caspase-3 cascade and down-regulating Bcl-2 and up-regulating Bax gene expressions as reported by Moradi, Heidari-Soureshjani, Asadi-Samani, and Yang (2017). Almond oil too shows antiproliferative and anticancerous activities as elucidated in colon cancer cell lines (Colo-320 and Colo-741) by MTT assay, this can be a promising therapeutic and preventive agent for cancers (Mericli et al., 2017).

11.5.35 *Raphanus raphanistrum* ssp. *sativus* (radish)

Both the roots as well as leaves of *R. rapahanistrum* ssp. *sativus* show antioxidant activity which is due to pyrogallol, vanillic acid, epicatechin and coumaric acid found in these as major polyphenols. An isothiocyanate sulforaphene has been isolated from its seeds which could be a candidate drug to cure some cancer types. According to Pawlik, Wała, Hać, Felczykowska, and Herman-Antosiewicz (2017) low doses of this compound show a pronounced antiproliferative activity against breast cancer cells by increasing autophagy, cell cycle arrest at G2, Bax:Bcl2 ratio and ADRP (adipose differentiation-related protein) levels. The nutritional values of this palnr are effected by cooking or any other thermal process and germination stage. Glucoraphenin level decreases with the predominant glucosinolate and sulforaphene together with anticancerous activities after maturity and cooking compared to fresh 3-day-old radish sprouts (Li, Song, et al., 2017).

11.5.36 *Secale cereal* (rye)

Alkylresorcinol, a phenol, is present in higher concentrations in the rye bran. Its pollens contain secaloside, a tumor inhibitory glycoside, which shows antitumor activity against S180 sarcoma cell line with IC_{50} values of 5 μg (Jaton et al., 1997). The phytoestrogens from this plant inhibit the cell proliferation significantly in trophoblast of Jeg3 tumor cells, but rye lignans inhibit progesterone and elevate estradiol production in these cell lines (Matscheski et al., 2006).

11.5.37 *Sesamum indicum* (sesame)

Sesame seeds as well as oil are phytochemically rich and possess a wide range of health benefits. Sesamol found in this plant is known to have potent antioxidant and anticancerous activity. The tumor is highly inhibited in mice skin by treatment with sesamol, as apoptosis is induced due to overexpression of proapoptotic proteins like Bcl-2 and Bax (Bhardwaj et al., 2017). According to Namiki (1995) lignans from sesame especially sesaminol shows a synergistic effect with tocopherols enhancing the antioxidant activity of tocopherols; acting as a potent drug against carcinogenesis. The antitumor activity of sesamin a lignin from sesame may have a significant role in mediating with various signaling pathways like activation of NF-κB, STAT3, JNK, ERK1/2, p38-MAPK, PI3K/AKT, caspase-3, and p53 (Majdalawieh, Massri, & Nasrallah, 2017). Growth inhibition is observed in human hepatocarcinoma cells, HepG2 after exposure to the white sesame seed extracts, the reason may be high content of phenolics present in the sesame (Lin et al., 2017). Sesamin extract contains phosphoproteins which show antileukemic activity inducing apoptosis via nuclear antigen H731 and CLIP-associating protein 2 isoform X25 (CLASP2) as reported by (Wannapruk, Paemanee, Roytrakul, & Tanyong, 2017).

11.5.38 *Solanum lycopersicum* (tomato)

Paur et al. (2017) have noted that prostrate cancer patients show significant reduction in PSA (prostrate specific antigen) due to tomato consumption. Overexpression of InsP5-ptase involved in phosphoinositol metabolism in MCF-7 cells is the reason for an induction of antiproliferative action (Alimohammadi, Lahiani, McGehee, & Khodakovskaya, 2017). The reason for high antiproliferation rate due to tomatoes may be because of the high depot of lycopenes in it. Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model lacking carotenoid cleavage enzymes, β-carotene 9',10'-oxygenase (BCO2) model show low cellular proliferation when treated with tomato (Tan et al., 2017). In-vitro gastric cell cancer growth inhibition has been seen in the cases using pectins as sour, raw tomatoes which decrease the activity of galectin-3, a protein known to increase cell-cell adhesions, metastasis and angiogenesis (Kapoor & Dharmesh, 2017). A prolonged consumption of tomatoes also curtails skin carcinogenesis as observed, reason being increase in concentration of lycopenes in skin and plasma after tomato consumption. The 2 glycoalkaloids tomatidine and hydroxylated-tomatidine are formed in skin after tomato exposure and probably protect skin against UV-induced keratinocyte carcinoma (Cooperstone et al., 2017).

11.5.39 *Solanum melongena* (brinjal)

The flavonoid from brinjal delphinidin cause growth inhibition in human fibrosarcoma HT-1080 cell lines (Nagase, Sasaki, Kito, Haga, & Sato, 1998). In these cells the cell invasion is decreased because delphidin inhibits activity of matrix metalloproteinase (MMP)-2 and MMP-9 which are responsible for tumor cell invasiveness. Phenolic derivatives from this plant act as potent antioxidants and show anticancer activity against several cancer cell lines (Sharma, 2017). According to Shabana, Salama, Ezzat, and Ismail (2013) methanol extract of *S. melongena* fruit peels has solasodine, solamargine and solasonine alkaloids together with 2 glycosides; β-sitosterol-3-*O*-β-D-glucoside; poriferasterol-3-*O*-β-D-glucoside which are responsible for dose-dependent reduction in proliferation in hepatocellular carcinoma cell lines, HEPG2. These cell lines show reduction in tumor marker, α-fitorotein and various histopathological changes.

11.5.40 *Solanum tuberosum* (potato)

S. tuberosum is rich in anthocyanins and phenolic acids, possessing high rate of antioxidant, antimicrobial and anticancerous activities, leading to cancer cell death by inducing apoptosis in cancer cell models like hematological and solid cancers in a dose-dependent manner as mentioned by Bontempo et al. (2013). Major anthocyanins are malvidin 3-*O*-*p*-coumaroyl-rutinoside-5-*O*-glucoside and petunidin 3-*O*-*p*-coumaroyl-rutinoside-5-*O*-glucoside which are responsible for its pharmacological activities. Chlorogenic acid; a polyphenol present in potato; is highly antioxidant with important antiproliferative activity in colon cancer and liver cell carcinomas (Wang et al., 2011). The data published by Madiwale, Reddivari, Holm, and Vanamala (2011) has revealed that after their storage the antioxidant activity increases but a decrease is seen in antiproliferative activity compared to fresh product as observed in human colon carcinomas. Potato anthocyanins have antiproliferative actions via apoptosis in both androgen receptor sensitive LNCaP and insensitive PC-3cancer prostrate cancer cell lines (Reddivari, Vanamala, Chintharlapalli, Safe, & Miller, 2007). In LNCaP cells caspase-dependent and caspase-independent pathways of apoptosis are induced whereas in PC-3 cells, only caspase-independent apoptosis pathway is induced via activation of nuclear translocation of endonuclease G (Endo G) and apoptosis-inducing factor (AIF), but inhibition of cyclin-dependent kinase inhibitor p27 is observed in both cell types.

11.5.41 *Sorghum* species (sorghum)

This plant is rich in tannins and flavonoids which show antioxidant, antiinflammatory, immunomodulatory and anticancerous activities. In traditional medicine *Sorghum* is basically used to cure anemia, inflammatory disease of liver, some viral diseases and leaf extract kills pain. Proanthocyanidins- as flavonoids are responsible for decreased proliferation and migration in hepatocarcinoma (HepG2) cell line by downregulating the p38 downstream signaling pathway of tumor progression as reported by Zhu, Shi, Yao, Hao, and Ren (2017). According to Chen, Rhodes, et al. (2017) its phenolic rich extract causes a dose-dependent cell cycle arrest at G2/M phase and cell inhibition in HepG2 liver carcinoma cells. The black, red and white varieties of sorghum are rich in 3-Deoxyanthoxyanins (3-DXA) which inhibit growth of HT-29 human colon cancer cells. Yang, Browning, and Awika (2009) have reported that dimethoxylated 3-DXA is more potent in inhibiting growth in colon cancer cells. The expression of antiapoptotic genes like survivin, cIAP-2,

XIAP, inducing apoptosis and cell death in human colon cancer cells are resulting from the anthocyanin present in this plant and show inhibitory action. Cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside anthocyanidins have potential inhibitory effect on tyrosine-kinases, causing antiproliferative effect on colon cancer cells (Mazewski, Liang, & de Mejia, 2018). In human breast cancer cells some important levels of apoptosis are seen following treatment with anthocyanins from red *Sorghum* bran (MCF-7) (Devi, Kumar, & Das, 2011), concentration dependent inhibition is observed and change in cellular architecture and formation of apoptotic bodies is quite significant. Park, Darvin, Lim, Joung, and Hong (2012) have shown that expression of VEGF through JAK/STAT signal transduction pathway in breast carcinomas is decreased by this plant, with cell cycle arrest in G1 phase and downregulation in the expression level of genes like cyclin D, cyclin E, and pRb in MDA-MB-231 breast cancer cell lines. Lipid derivatives like tocopherols (predominantly γ-tocopherol), triacylglycerides (particularly linoleic acid), policosanols, aldehydes, and sterols (particularly campesterol and stigmasterol) from this plant are responsible for a significant inhibition in growth of human epithelial colorectal carcinoma cell line (caco-2) (Zbasnik et al., 2009).

11.5.42 *Trigonella foenum-graecum* (fenugreek)

The findings of Raju and Mehta (2009) have revealed that diosgenin acts as an inhibitor for COX enzymes with possible effects on 5-LOX activity. On the other hand Li, Fernandez, Rajendran, Hui, and Sethi (2010) have reported that diosgenin shows a mediating role in STAT3 signaling pathway in hepatocellular carcinoma by suppression of the activation of c-Src, JAK1, and JAK2. Extract of *T. foenum-graecum* increases apoptosis, ethanolic extract from dry plants induces cell death in human T-lymphoma Jurkat cells (Al-Daghri et al., 2012; Khoja et al., 2011). The cytotoxic effects of extract of this plant have been seen in some cancer cell lines, stressing the usefulness of this plant for antineoplastic activity (Alsemari et al., 2014).

11.5.43 *Triticum* species (wheat)

Wheat Grass Juice is rich in bioflavonoids, minerals and several essential amino acids. It has high therapeutic efficacy and helps in the reduction in dose and myelotoxicity, increasing the prognosis in patients undergoing therapy. The patients suffering from myelodysplasia and decrease in the number of transfusions the juice can reduce serum ferritin via iron chelation. According to Tandon, Arora, Singh, Monga, and Arora (2011) its extract decreases benzopyrene-induced mutagenicity and has antiproliferative action in breast cancer cell lines, MCF-7 because of high antioxidative properties. Ki, Poudel, Lee, Lee, and Kim (2017) have reported that dichloromethane fraction of wheat extract can be used to treat several cancer types because it can suppress the growth of melanoma cells to an extent similar to cisplatin. Wheat grass helps in the prevention of myelotoxicity in patients undergoing chemotheraphy due to its anticlastogenic and cytoprotective effects.

11.5.44 *Vigna unguiculata* (cowpea)

This species is rich in vitamin C, phytates and phenols (Ashraf et al., 2016; Ozturk, Gemici, & Guvensen, 1993). All these are responsible for its antioxidant and anticancerous activities. The phenolic extract of whole seeds decreases growth of mammary cancer cell, MCF-7 up to 65% (Gutiérrez-Uribe, Romo-Lopez, & Serna-Saldívar, 2011). *V. unguiculata* seed coats inhibit cancer cell growth, but inhibition is lesser as compared to the whole seed extract. Its seeds are rich in flavonoids like kaempferol, and terpenes like stigmasterol which inhibit formation of many cancers. Kaempferol is a strong antioxidant and repairs the damage to lipids, proteins and DNA originating from cancer metastasis (Battu, Ckvlsn, Priya, Malleswari, & Reeshm, 2011).

11.5.45 *Vitis vinifera* (grapes)

Grapes contain many resveratrol in natural amalgamation with catechins, anthocyanins, polyphenols and flavonols. High amount of polyphenols is responsible for the antioxidative and antiinflammmatory characteristics and can as such be used as chemotherapeutic and chemoprotective agent. According to Zaineddin et al. (2012) treatment with seed and skin extracts of muscadine (*Vitis rotundifolia*) in human breast carcinoma cell line, SKBR3 HER2, a decrease in proliferation occurs due to variety of grapes in a dose-dependent manner, affecting AKT-mTOR downstream signaling pathway. Muscadine variety of grapes decreases growth of LnCaP prostrate cancer cell lines and angiogenesis by downregulating the expression of placental growth factor (PLGF) and vascular endothelial growth factor (VEGF) as

reported by Gallagher and Tallant (2017). High inhibition has been observed in the growth of triple breast cancer cell lines MDA-MB-231, BT-549 and BT-20 of human and 4T1 murine cells. Decrease in skin tumorigenesis in rats has been recorded due to grape powder subjected to UV-B rays by causing a significant decrease in Ki67 and PCNA proliferation markers (Singh, Ndiaye, Mintie, Chhabra, & Ahmad, 2017). Grape powder probably helps in the chemoprevention of various cancers. Procyanidin rich fraction of grapes leads to a time-dependent decrease in cell proliferation in 2 cancer cell lines, HeLa and BCPAP as mentioned by Ghouila et al. (2017). A significant antitumor activity has been seen following use of grape seed extract encapsulated in chitosan microparticles with increased cellular interactions. Tons of grape pomace; left over after making wine; are source of many compounds of medicinal value. According to Luo et al. (2017) it inhibits growth of MDA-MB-231 and MCF-7 breast cancer cell lines by inducing apoptosis and cell cycle arrest at S-phase.

11.5.46 *Withania somnifera* (ashwagandha)

W. somnifera has been studied at length against the breast cancer. Inhibitory activities in various cell lines have been recorded. The cell cycle arrest too has been established leading to the apoptosis as the chief mechanism of action (Achar et al., 2018; Maliyakkal, Udupa, Pai, & Rangarajan, 2013). Its extract inhibits cell proliferation in breast cancer cell lines with significant reduction in the cytokine and CCL2 expression (Khazal & Hill, 2015).

According to Senthilnathan, Padmavathi, Magesh, and Sakthisekaran (2006) and Achar et al. (2018) ashwagandha extract together with paclitaxel stabilizes membrane-bound enzyme levels and decreases lipid peroxidation involving molecular inhibitory mechanism in the chemotherapeutic activities. This plant has also been reported to possess anticancer activity against prostate cancer, colon, lung carcinoma, and liver cancer, leukemia, skin cancer, and head and neck cancer of various human carcinomas as reported by Achar et al. (2018), Aalinkeel et al. (2010), and Mathur et al. (2006) respectively. Ashwagandha seems to play an effective role in cancer prevention (Achar et al., 2018; Palliyaguru, Singh, & Kensler, 2016). It has shown antitumor effect on transplantable tumor models, metastatic skin cancer cell lines (Achar et al., 2018).

11.5.47 *Zea mays* (maize)

Important medicinal characteristics have been reported for maize because of the wide range of its bioactive peptides, which are good for hepatoprotection, in antihypertension, as antioxidant, and antitumorogen. Protein hydrolysates of maize have decreased the growth of cancer cell lines like HepG2 and H22 tumor-bearing mice in in vitro models (Li et al., 2013). Blue corn; as pigmented maize; is a rich source of polyphenols especially anthocyanins which show highly anticancerous. Its extract inhibits the growth of human breast cancer (MCF-7), lung cancer (SKLU-1), myelogenous leukemic (K562), prostate cancer (PC-3) and colon cancer (HCT-15) cell lines in a dose-dependent manner with highest inhibition at a dose of 500 μg/mL (Guzmán-Gerónimo, Aparicio, Barradas, Chávez-Servia, & Alarcón-Zavaleta, 2017). Biopeptides of this plant decrease the expression of antiapoptotic signals leading to cell death in liver carcinomas (HepG2 cells) (Ortiz-Martinez et al., 2017).

11.5.48 *Zingiber officinale* (ginger)

Different bioactive phenolics such as; gingerols (6-gingerol), paradols, and shogaols are found in *Z. officinale* (Karna et al., 2012; Siddiqui et al., 2018). As per Zick et al. (2009) promising results have been recorded with encapsulated ginger, used as a treatment for chemotherapy-induced nausea and vomiting. Other studies are on the way to define exactly the use of ginger in cancer management (Siddiqui et al., 2018). Crude ginger ethanolic extract has shown cytotoxicity and anticancer activity against cholangiocarcinoma (Plengsuriyakaran et al., 2012). Anticancer activity of ginger leaf has been investigated by Park et al. (2014) under in-vitro condition. They found reduction in cell viability and apoptosis in human colorectal cancer cells.

11.6 Important potential anticancer exotic plants

11.6.1 *Calendula officinalis* (marigold)

This is rich in flavonol glycosides, carotenoids, saponins triterpene oligoglycosides, oleanane-type triterpene glycosides, and a sesquiterpene glucoside. Jiménez-Medina et al. (2006) have reported that an aqueous extract using Laser Activated *Calendula* Extract (LACE) inhibits tumor cell proliferation of a wide variety of cancer cells both in vivo and

in vitro. The possible reason for anticancerous activity in the cell lines for an arrest of cell cycle in G0/G1 phase and activation of Caspase-3-induced apoptosis seems to be due to this plant extract. Oral administration of this extract has also inhibited the growth of Ando-2 melanoma cells injected intraperitoneally in nude mice. According to Ukiya et al. (2006) two triterpene glycosides extracted from its flowers show potent cytotoxic effects against colon cancer, leukemia, and melanoma cells with simultaneous antiinflammation in 2-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation.

11.6.2 *Eucalyptus* species (gum tree)

The triterpenoic acids "ursolic and oleanolic" are obtained from this species by using methanol and dichloromethane solvents. Al-Marzook and Omran (2017) have reported that alkaloid rich extract of this species has resulted in a 45.25% and 92% reduction in the cell viability of MCF-7 and WRL-68 cell lines respectively; however, there is need for a deeper study on its mechanism. An aqueous extract of leaf and ethanolic extract of fruit of *E. microcorys* causes almost 80% growth inhibition in glioblastoma, neuroblastoma, lung and pancreatic cancer cells at 100 μg/mL (Bhuyan et al., 2017). After 24 h of treatment with the extracts, transcriptional activation of Caspase 3/7 leads to apoptosis in pancreatic cancer (PaC-2) cell lines. Its methanolic extracts are responsible for the cytotoxicity in P19 embryonal carcinoma cell, cancerous stem cells. They can help in alleviating tumor recurrence due to multidrug resistance (Soltanian, Sheikhbahaei, & Mohamadi, 2017). According to Hrubik et al. (2012) different extracts of *Eucalyptus camaldulensis* lead to cytotoxicity in human breast cancer cell lines (MCF-7 and MDA-MB-231) as found after using SRB and MTT assays.

11.6.3 *Robinia pseudoacacia* (black locust)

An ethanolic extracts of whole plant has revealed the presence pharmacologically important 5 bioactive flavonoids namely; acacetin, secundiflorol I, mucronulatol, isomucronulatol, and isovestitol. All these cause cytotoxicities in 6 solid tumor cell lines in humans but, acacetin shows highest cytotoxicity in prostate cancer cell line (PC-3) (Tian & McLaughlin, 2000).

11.6.4 *Sambucus nigra* (elder)

Good chemopreventive potential has been observed in the aqueous extracts which give the anticancerous name to this plant. It contains phytocompounds such as; flavonoids, sesquiterpenes, iridoid monoterpene glycosides, and phytosterols which are anticancerous. Thole et al. (2006) have reported that the extract of this plant induces some enzymes like quinone is rich in anthocyanins and other flavonoids which are antioxidant, anticarcinogenic, immunomodulatory, antibacterial in nature and contribute in the prevention of various degenerative diseases like cancer (Akbulut, Ercisli, & Tosun, 2009). A significant inhibition of human colon adenocarcinoma cell line (LoVo) and breast cancer cell line (MCF-7) proliferation is seen after treatment with aqueous acetone solution (Gleńsk et al., 2017). Ursolic acid as a triterpenoid shows the highest activity against the carcinoma cell lines with IC_{50} values of 10.7 μg/mL on MCF-7 and 7.7 μg/mL on LoVo cells. Extracts of flower and berries inhibit cell proliferation of MCF-7 and BT20 cells. This is due to the phytocompounds like quercetin, chrysin, kaempferol, naringenin and hesperidin when applied in different doses (Stapel, Oppermann, Richter, Ruth, & Briese, 2013).

11.7 Other alternative plants

Some plants of natural, cultural and exotic origin in the Himalayas are mentioned to show anticancer activity as per the local residents. These plants are *Amaranthus spinosus*, *Azadirachta indica*, *Celtis australis*, *Cupressus torulosa*, *Dillenia indica*, *Dillenia pentagyna*, *Fragaria nubicola*, *Geranium wallichianum*, *Gloriosa superba*, *Hypericum perforatum*, *Indigofera tinctoria*, *Iris nepalensis*, *Marrubium vulgare*, *Rorippa sylvestris*, *Skimmia laureola*, *T. baccata*, and *Xanthium strumarium*. There is a need for their evaluation and investigation of active substance should be analyzed followed by clinical studies. These plants must be studied in detail on cancer cells in vivo or in vitro.

11.8 Conclusions

Cancer is accepted as a dangerous disease with mortality rates increasing at a higher rate in spite of huge investments on research and development of new drugs for its prevention and cure. The methods used at present include surgery, chemo and radiation therapies. However, these have limitations because of their toxic effects on nontargeted cells leading to side effects on our health problems. As stressed by De Cicco et al. (2018) we must look for alternative treatments with natural anticancer agents from plants. The reports show that at present 60% of the cancer drugs available in the market or in testing are based on natural products. Out of 177 anticancer drugs approved more than 70% are from natural products or mimetic. Nearly 25% of the prescription drugs in the world market come from the plants and almost 121 of these are in use. In different countries clinical trials are in progress with over 100 natural product-based drugs. Almost 11% of 252 drugs found in the WHO essential list of medicines are of plant origin (De Cicco et al., 2018).

Anticancer drugs obtained from the compounds originating from plants play an important role in the cancer treatment. These show good immunomodulatory and antioxidant features with anticancer activity (Tan & Norhaizan, 2018). The plant life on our earth, especially those distributed in the Himalayas, represents an enormous diversity. They contain potential bioactive components against a large number of diseases, including cancer. Their potential implication could be significant to replace conventional therapies, but should be evaluated well in long-term clinical trials (Tan & Norhaizan, 2018).

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Chapter 12

Diversity and bioprospect significance of macrofungi in the scrub jungles of southwest India

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12.1 Introduction

Fungi are a diverse group of organisms that are becoming extremely important in several vital ecological services, especially nutrient cycles, carbon sequestration, life-saving metabolites, production of biofuels, and degradation of pollutants. Although fungi have been used in human nutrition and medicine over the past 6000 years, they were hidden behind the shadows of plants and animals (Cannon, Aguirre-Hudson, & Aime, 2018). In spite of early investigations that considered fungi as lower plants, recent molecular diagnostics has confirmed their position as a kingdom which has more affinity to animals than plants (Baldauf, Romeralo, & Carr, 2013). The recent report, *State of the World's Fungi 2018* provides updated information on diversity, plant−fungal interactions, and the necessities of conservation of fungi (Willis, 2018). Based on the angiosperm−fungus ratio, the currently accepted global estimate of total fungi ranges from 2.2 to 3.8 million (Hawksworth & Lücking, 2017). Mueller, Schmit, and Leacock (2007) estimated the range of macrofungal diversity as 53,000−110,000. This estimate has been revised up to 0.22−0.38 million considering 10% of global fungi belong to macrofungi (Hawksworth, 2019; Rossman, 1994).

The coastal regions of the Indian Subcontinent have been considered as one of the 10 biogeographic zones (Singh & Chaturvedi, 2017). The scrub jungles established on the valleys and mountains of the southwest coast of India consist of short crown thorny xeric shrubs with grasslands on the lateritic substrate. These scrub jungles are the sites of macrofungi owing to occurrence of diverse flora and the deposition of various recalcitrant debris (e.g., leaf litter, woody litter, bark, inflorescence, and flowers). In addition, several habitats support macrofungi in scrub jungles, including termite mounds, live roots, seeds, herbivore dung, and insects. Along with scrub jungles, the coastal region also encompasses sacred groves, plantations, botanical gardens, and avenue trees. To understand the species richness and diversity of fungi in any ecosystem, the first and foremost endeavor is to perform several expeditions. Accordingly, a few surveys have been carried out on macrofungi of the scrub jungles of southwest Karnataka (Greeshma, Sridhar, Pavithra, & Ghate, 2016; Karun & Sridhar, 2014; Pavithra, Sridhar, Greeshma, & Karun, 2016). Studies on macrofungi in these scrub jungles were initiated in 2011, and these revealed an increased population consisting edible, medicinal, and ectomycorrhizal fungi (Sridhar, 2018a). However, the richness and diversity of macrofungi in the scrub jungles are less than in the Western Ghats (Karun & Sridhar, 2016) due to climatic conditions (e.g., altitude, temperature, and rainfall), forest cover, and biotic diversity. There will be an almost steep decline of the occurrence of macrofungi in scrub jungles from the southwest monsoon to postmonsoon, while in the Western Ghats it will be extended beyond the monsoon season (Karun & Sridhar, 2014, 2016). So far from the scrub jungles of Karnataka, 89 species belonging to 59 genera have been reported (Sridhar, 2018b). The purpose of this chapter is to assess the macrofungal assemblage in fairly undisturbed scrub jungles of southwest India to emphasize their biodiversity and economic significance.

12.2 Study area

The geographic location (Mangalore University Campus) chosen for the macrofungal survey was located ∼5−8 km from the Arabian Sea coast, adjacent to Mangalore City (Dakshina Kannada, Karnataka) (12°48′ N, 74°55′E;

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104−112 m asl). The vegetation in this undulating terrain (~150 ha) was grassland and developed patches of scrub jungles of about five decades. The natural scrub vegetation builds up gradually and the plantation of cashew (*Anacardium occidentale*) was established in the early 1970s. Subsequently, the forest department has taken an interest in developing mixed plantations (e.g., *Acacia*, *Anacardium*, *Azadirachta*, *Casuarina*, *Cocos*, and *Pongamia*). The National Service Scheme (NSS) of college students have served to develop the native natural vegetation of this region. Several tree species have built up subsequently without human involvement (e.g., *Borassus*, *Careya*, *Caryota*, *Holigarna*, *Hopea*, *Macaranga*, *Sapium*, *Syzygium*, *Tamarindus*, and *Terminalia*). In addition, adjacent to the study area are many ethnically conserved sacred groves of scrub jungles called "Naga Bana" or "Nagara Bana" (Kannada vernacular: Naga = *Naja*; Bana = Forest), which are almost devoid of human interference. In addition, each agricultural land will have certain areas of scrub jungles to cater for the needs of green manure.

12.3 Survey and data analysis

The macrofungal survey was carried out in 12 weeks during the southwest monsoon season (June−August 2018). Humidity (under the tree), air temperature (below the canopy), and soil temperature (5−10 cm depth) were measured during each survey (Mextech Digital Thermo Hygrometer M288CTHW, Mextech Technologies India Private Limited, Mumbai, Maharashtra, India). Depending on the rain conditions and extent of sporocarps, the survey was carried out during morning (8−11 a.m.) and or evening (4−7 p.m.) hours on each week in fairly undisturbed locations of the scrub jungles, spread over 150 ha. On each sampling date, a quadrat of 25 m^2 was marked on the ground to screen sporocarps of macrofungi on different substrates (soil, leaf litter, woody litter, live roots, seeds, and insects). Characteristic features of sporocarps was noted on the sampling site, specific sporocarps were blotted and brought to the laboratory within 1 or 2 h for further study and preservation. Examination of each species was carried out for diagnostic features by magnifying lens and microscope (Nikon YS100, Nikon Corporation, Tokyo, Japan) (Aravindakshan & Manimohan, 2015; Buczacki, 2012; Cannon & Kirk, 2007; Jordan, 2004; Mohanan, 2011; Pegler, 1990; Phillips, 2006; Wannathes, Desjardin, & Hyde, 2009). The specimens were oven-dried (55°C−60°C) to preserve in dry conditions and preserved in a mixture of water−ethanol−formaldehyde (14:5:1).

Based on the number of sporocarps of each species in 12 quadrats, sporocarps per quadrat, and relative abundance (%) were calculated. The diversity (Simpson and Shannon diversities) and equitability (Pielou's evenness) of macrofungi were determined (Magurran, 1988; Pielou, 1975).

12.4 Richness, diversity, and survey interval

During the period of study, the humidity, air temperature, and soil temperature ranged from 71% to 89%, 22.8°C to 29.0°C, and 24.0°C to 27.9°C, respectively (Table 12.1). The present study disclosed the occurrence of 90 species belongs to 45 genera in scrub jungles (Table 12.2; Fig. 12.1). The most common genus was *Marasmius* (14 species) followed by *Cordyceps* (6 species), *Mycena* (5 species), *Xylaria* (5 species), *Hygrocybe* (4 species), and the remaining 11 genera had less than three species each. *Marasmius haematocephalus* was the most dominant followed by 11 core group species (>5 mean sporocarps/quadrat) (*Geastrum triplex*, *Lenzites betulinus*, four species of *Marasmius*, *Mycena adscendens*, *Tetrapyrgos nigripes*, and three species of *Xylaria*). The species richness showed a progressive increase with three peaks during the 3rd, 6th, and 8th weeks with the highest richness in the 8th week, and thereafter it dropped abruptly (Fig. 12.2A). The sporocarp richness showed two peaks during 3rd and 7th week with the highest richness in the 7th week and thereafter it dropped abruptly (Fig. 12.2B). The cumulative species, as well as cumulative sporocarps, reached saturation after 10 weeks. The diversity progressively increased and attained a peak during the 8th week as seen in species richness, while the Pielou's equitability showed three peaks during the 4th, 8th, and 11th weeks with the highest in the 11th week (Fig. 12.2C). The overall Simpson and Shannon diversities were 0.961 and 5.281, respectively, while the Pielou's equitability was 0.972. Among the macrofungi, the highest number of species were medicinal (27.8%) followed by edible (20%) and ectomycorrhizal (6.7%) (Fig. 12.3).

A few surveys have been carried out in the region of lateritic scrub jungles of southwest India (e.g., Karun & Sridhar, 2014; Pavithra et al., 2016). Such surveys are confined to plantations, secondary forests, and botanical gardens by fortnightly or monthly intervals. The present study was carried out exclusively in patches of fairly undisturbed natural scrub jungles located in different regions at weekly intervals, which has resulted in higher species richness as well as diversity compared to earlier surveys (Table 12.3). Assessment of individual trees (*A. occidentale* and *Terminalia paniculata*) in and around scrub jungles on weekly intervals revealed the occurrence of a lower number of macrofungi (22−36 vs 90 species) as well as diversity compared to the present study (Jagadish, Sridhar, & Dattaraj, 2019). Another

TABLE 12.1 Range of abiotic conditions of scrub jungle during macrofungal survey (June–August 2018).

	Humidity (%)	Temperature (°C)	
		Air	Soil
June 03–09	81–82	25.0–27.0	24.0–25.3
June 10–16	80–84	26.1–29.0	25.9–26.0
June 17–23	72–87	26.5–27.0	26.7–27.3
June 24–30	84–86	24.9–25.4	25.3–26.2
July 01–07	85–87	26.0–27.0	25.8–26.3
July 08–14	72–83	22.8–26.6	24.8–26.0
July 15–21	83–87	26.8–28.0	25.0–27.2
July 22–28	71–81	26.0–27.4	26.8–27.1
July 29–August 04	85–89	26.8–27.0	25.7–26.2
August 05–11	83–86	25.0–25.9	24.0–26.1
August 12–18	78–86	24.0–26.3	25.5–27.0
August 19–25	75–86	26.0–29.0	26.0–27.9

fortnightly study confined to scrub jungles, however, revealed a higher number of species as well as Shannon diversity compared to the plantations and individual trees (Greeshma et al., 2016). This pattern of occurrence shows the possibilities of supporting high species richness and diversity of macrofungi owing to the mosaic of native vegetation in scrub jungles. In addition, our study revealed that macrofungi in scrub jungles will sustain for a short period (~3 months), thus a short period of expedition (once or twice a week) will allow the scoring of many fragile species that show as sporocarps for only a short period (e.g., *Marasmius* spp.). In addition, the abiotic factors vary from June to August, with an increase in temperatures (air and soil) and a decrease in atmospheric humidity, which might influence the macrofungal sustenance in scrub jungles.

Earlier studies in and around scrub jungles consist of a single peak of species and sporocarp richness either during June or July (Karun & Sridhar, 2014; Pavithra et al., 2016). Owing to the weekly survey in our study, the macrofungi showed multiple peaks with one highest peak of species richness, sporocarp richness, and diversity during July, which is similar to another study in scrub jungles (Greeshma et al., 2016). Such a difference might be due to surveys being performed in secondary forests or botanical gardens, differences in sampling interval, the substrates assessed, and the extent of abiotic factors. Unlike in the Western Ghats (Karun & Sridhar, 2016), the sporocarp richness of macrofungi was saturated over about 10 weeks duration in scrub jungles reflecting the impact of abiotic factors.

12.5 Core group fungi

Compared to earlier studies, the composition of the core group of fungi differed substantially in the present study (Table 12.4). Thirty two species belong to the core group in and around scrub jungles. Of the total core group species 16 were in plantations, while 21 were in the scrub jungles. Eleven core group species were confined to plantations, 16 were confined to the scrub jungles, and only five species were in common. Interestingly, among the 12 economically valuable core group fungi (edible, medicinal and ectomycorrhizal), 10 were confined to the scrub jungles (*Astraeus odoratus*, *Cyathus striatus*, *Dacryopinax spathularia*, *Lentinus squarrosulus*, *L. betulinus*, *Phallus indusiatus*, *Pycnoporus sanguineus*, *Schizophyllum commune*, *Thelephora palmate*, and *Xylaria polymorpha*), while only two species were in common (*G. triplex* and *Xylaria hypoxylon*).

12.6 Substrate preference

Four major substrates, such as wood, soil, leaf, and insect, supported the growth of macrofungi (Fig. 12.4). The woody substrates were preferred by the highest number of macrofungi (46.7%), followed by soil (36.7%), leaf litter (14.4%),

TABLE 12.2 Assemblage of macrofungi in scrub jungles.

	MS	RA (%)	Substrate
Marasmius haematocephalus (Mont.) Fr.	18.5	8.7	L
Tetrapyrgos nigripes (Fr.) E. Horak	16.8	7.9	T, W
Marasmius guyanensis Mont.	12.3	5.8	L
Marasmius rotula (Scop.) Fr.	10.9	5.2	T
Marasmius katangensis Singer	10.8	5.1	T
Xylaria hypoxylon (L.) Grev.[b]	9.8	4.6	S, W
Marasmius sp. 3 (Fig. 12.1Q)	9.4	4.5	L
Xylaria polymorpha (Pers.) Grev.[b] (Fig. 12.1X)	9.1	4.3	W
Mycena adscendens Mass Geest.	8.8	4.2	L, W
Geastrum triplex Jungh.[b,c]	8.0	3.8	R
Lenzites betulinus (L.) Fr.[b]	5.6	2.6	W
Xylaria minuta Panwar	5.2	2.5	T
Microporus vernicipes (Berk.) Kuntze	4.8	2.3	W
Pycnoporus cinnabarinus (Jacq.) P. Karst.[b]	4.3	2.1	W
Dacryopinax spathularia (Schwein.) G.W. Martin[a] (Fig. 12.1J)	4.3	2.0	W
Ramaria versatilis Quél.[a]	3.7	1.7	S
Xylaria escharoidea (Berk.) Sacc. (Fig. 12.1W)	3.7	1.7	Tm
Auricularia auricula (L.) Underw.[a]	3.4	1.6	W
Clathrus delicatus Berk. & Broome	2.9	1.4	W
Nectria cinnabarina (Tode) Fr.	2.9	1.4	W
Marasmius oreades (Bolton) Fr.[a,b]	2.7	1.3	L
Tremella reticulata (Berk.) Farl.[a]	2.6	1.2	W
Hygrocybe astatogala R. Heim ex Heinem.	2.4	1.1	S
Daldinia concentrica (Bolton) Ces. & De Not.[b] (Fig. 12.1K)	2.3	1.1	W
Marasmius crinipes Antonin, Ryoo & H.D. Shin	2.2	1.0	L
Omphalotus olearius (DC.) Singer[b] (Fig. 12.1R)	2.0	1.0	W
Marasmius sp. 2	1.9	0.9	L
Marasmius crinis-equi F. Muell. ex Kalchbr.[a]	1.9	0.9	L
Marasmius sp. 1	1.8	0.9	L
Xylaria longipes Nitschke[b]	1.6	0.8	W
Entoloma serrulatum (Fr.) Hester	1.5	0.7	S
Amauroderma conjunctum (Lloyd) Torrend[b]	1.3	0.6	S, W
Coprinus plicatilis (Curtis) Fr.[a] (Fig. 12.1B)	1.3	0.6	S
Laccaria fraterna (Sacc.) Pegler[c]	1.3	0.6	R
Lenzites vespaceus (Pers.) Pat.	1.3	0.6	W
Leucoagaricus sp. 1 (Fig. 12.1P)	1.2	0.6	S
Marasmius dendrosetosus Shay & Desjardin	1.2	0.6	W
Lepiota sp. 1	1.2	0.6	S

(*Continued*)

TABLE 12.2 (Continued)

	MS	RA (%)	Substrate
Ramaria pallida Marie (Fig. 12.1S)	1.1	0.5	S, L
Hygrocybe alwisii (Berk. & Broome) Pegler[a] (Fig. 12.1M)	1.1	0.5	S
Marasmius spegazzinii (Kuntze) Sacc. & Syd.	1.0	0.5	T
Cyathus striatus (Huds.) Willd.[b]	0.9	0.4	Se
Marasmiellus sp.	0.9	0.4	T
Clathrus sp.	0.8	0.4	T
Geastrum lageniforme Vittad.[c] (Fig. 12.1L)	0.8	0.4	S, R
Marasmius androsaceus (L.) Fr.[b]	0.8	0.4	W
Ganoderma lucidum (Curtis) P. Karst.[b]	0.8	0.4	W
Hexagonia tenuis (Fr.) Fr.	0.7	0.3	W
Hygrocybe conica (Schaeff.) P. Kumm.[a] (Fig. 12.1N)	0.7.	0.3	S
Marasmiellus ignobilis (Berk. & Broome) Pegler	0.7	0.3	T
Mycena sp. 1	0.7	0.3	L
Coprinus sp.	0.6	0.3	S
Conocybe apala (Fr.) Arnolds	0.5	0.2	S
Conocybe crispa (Longyear) Singer	0.5	0.2	S
Scleroderma sp.[c]	0.5	0.2	R
Marasmius sullivantii Mont.	1.4	0.2	T
Phellinus gilvus (Schwein.) Pat.[b]	0.4	0.2	B, W
Tremella fuciformis Berk.[a,b]	0.4	0.2	T
Termitomyces fuliginosus R. Heim[a]	0.4	0.2	Tm
Astraeus odoratus Phosri[a,c] (Fig. 12.1A)	0.3	0.2	R
Cookeina tricholoma (Mont.) Kuntze[b] (Fig. 12.1C)	0.3	0.2	T, W
Cordyceps sp. 1[b]	0.3	0.2	Gh
Mycena sp. 2	0.3	0.2	L
Agaricus sp. 1	0.3	0.1	S
Amylosporus campbellii (Berk.) Ryvarden[a,b]	0.3	0.1	W
Entoloma anamikum Manim., A.V. Joseph & Leelav.	0.3	0.1	S
Hygrocybe sp.	0.3	0.1	S
Geastrum rufescens Pers.	0.3	0.1	S
Leucoagaricus sp. 2	0.3	0.1	S
Schizophyllum commune Fr.[a,b] (Fig. 12.1T)	0.3	0.1	W
Xerula sp.	0.3	0.1	S
Mycena chlorophos (Berk. & M.A. curtis) Sacc.	0.3	0.1	T
Amanita sp.[a,c]	0.2	<0.1	R
Chlorophyllum molybdites (G. Mey.) Massee[a]	0.2	<0.1	L
Cordyceps militaris (L.) Fr.[b] (Fig. 12.1D)	0.2	<0.1	Co

(*Continued*)

TABLE 12.2 (Continued)

	MS	RA (%)	Substrate
Cordyceps sp. 2[b] (Fig. 12.1E)	0.2	<0.1	La
Gymnopus dryophilus (Bull.) Murill	0.2	<0.1	S
Lentinus squarrosulus Mont.[a] (Fig. 12.1O)	0.2	<0.1	W
Lepiota sp. 2	0.2	<0.1	S
Mycena rosea Gramberg	0.2	<0.1	S
Phallus indusiatus Schitdl.[a,b]	0.2	<0.1	S
Agaricus sp. 2	0.1	<0.1	S
Cordyceps sp. 3[b] (Fig. 12.1F)	0.1	<0.1	La
Cordyceps sp. 4[b] (Fig. 12.1G)	0.1	<0.1	Co
Cordyceps sp. 5[b] (Fig. 12.1H)	0.1	<0.1	I
Cystoagaricus trisulphuratus (Berk.) Singer (Fig. 12.1I)	0.1	<0.1	S
Entoloma sp.	0.1	<0.1	S
Lepiota echinella (Quél. & G.E. Bernard)	0.1	<0.1	S
Termitomyces umkowaan (Cooke & Massee) D.A. Reid[a] (Fig. 12.1U)	0.1	<0.1	S, Tm
Trichoglossum hirsutum (Pers.) Boud. (Fig. 12.1V)	0.1	<0.1	S

MS, Mean sporocarps per quadrat; *RA*, relative abundance; Substrate: B, bark; Co, cocoon; Gh, grass hopper; I, insect; L, leaf litter; La, larva; R, root; S, soil; Se, seed; T, twig; Tm, termite mound; W, wood log/stub.
[a]*Edible.*
[b]*Medicinal.*
[c]*Ectomycorrhizal.*

and insects (6.7%). Recognizable woody debris comprised bark, live roots, twigs, live seeds, and logs. Similarly on leaf litter, macrofungi were seen on the lamina, veins, midrib, and petiole of a variety of leaves. In different transacts, macrofungi were growing on the lateritic soil composed of pebbles, gravel, loam, sandy loam, humus, termite mounds, and soils mixed with recognizable wood and or leaf pieces. The insect remains comprised dead grass hoppers, unidentified insects, larvae, and cocoons that were buried (~2–3 cm) in the soil.

The scrub jungles supply a variety of substrates as well as provide many ecological niches for macrofungi. However, woody debris showed the highest macrofungi compared with other substrates (46.7% vs 6.7%–36.7%) (Fig. 12.4). Such differences may be due to enrichment by scrub jungles by yielding different substrates (e.g., bark, twigs, logs, roots, leaf litter, inflorescence, and seeds). Among them, live roots and seeds harbor several mutualistic fungi (e.g., ectomycorrhizal and endophytic fungi) (Table 12.2). For instance, *C. striatus* was recorded on unknown live seeds in the present study, while *X. hypoxylon* was recorded on live unidentified seeds (unpublished observation) showing their endophytic association. Moreover, the scrub jungles support termite mounds as well as anthills. Two *Termitomyces* and one *Xylaria* species were confined to the termite mounds. Similarly, the diversity of insect fauna in scrub jungles was reflected in the occurrence of six *Cordyceps* species, whereas no such insect-inhabiting fungi have been reported in botanical gardens or in secondary forests. Substrate and niche preferences of macrofungi in scrub jungles seem to vary from those of plantations and secondary forests.

12.7 Noteworthy fungi

This study looks at several interesting macrofungi (~46%) based on traditional knowledge and FAO-WHO classification (Boa, 2004). In addition, several macrofungi are new to scrub jungles and many of them are edible (25 species), medicinal (18 species), and ectomycorrhizal (6 species) (Table 12.2). Based on traditional knowledge, none of them are used for medicinal purposes by the local dwellers, however, six edible macrofungi are ethnically preferred (e.g.,

FIGURE 12.1 Sporocarps of selected macrofungi: *Astraeus odoratus* (A), *Cookeina tricholoma* (B), *Coprinus plicatilis* (C), *Cordyceps militaris* (D), *Cordyceps* sp. 2 (E), *Cordyceps* sp. 3 (F), *Cordyceps* sp. 4 (G), *Cordyceps* sp. 5 (H), *Cystoagaricus trisulphuratus* (I), *Dacryopinax spathularia* (J), *Daldinia concentrica* (K), *Geastrum lageniforme* (L), *Hygrocybe alwisii* (M), *Hygrocybe conica* (N), *Lentinus squarrosulus* (O), *Leucoagaricus* sp. 1 (P), *Marasmius* sp. (Q), *Omphalotus olearius* (R), *Ramaria pallida* (S), *Schizophyllum commune* (T), *Termitomyces umkowaan* (U), *Trichoglossum hirsutum* (V), *Xylaria escharoidea* (W), and *Xylaria polymorpha* (X).

Amanita sp., *A. odoratus*, *P. indusiatus*, *L. squarrosulus*, and two *Termitomyces* species). The *A. odoratus* as well as *P. indusiatus* will be preferred in "egg-stage" (before basidiocarp ruptures), while *Amanita* sp. is preferred in tender stages (spherical or just opened dumbbell stage). *Termitomyces* species will be chosen based on their occurrence in termite mounds with long stripes, while the *L. squarrosulus* will be recognized by the characteristic pileus grown on logs. The local people are watchful for the growth of these edible macrofungi in the scrub jungles for harvesting purposes. Many edible macrofungi are consumed in the immature "egg-stage" (e.g., *Amanita*, *Astraeus*, and *Phallus*); there are likely some truffles existing beneath the soil of these scrub jungles that require precise study for confirmation through ethnic familiarity.

Among the six ectomycorrhizal fungi found in scrub jungles, confirmation for *G. triplex* was possible for its association with live roots of *T. paniculata*. For the remaining five species, although found in scrub jungles and associated with roots, the specific tree or shrub species could not be ascertained. For entomopathogenic fungi, the host of *Cordyceps* sp. 1 has been identified as a grass hopper, while the remaining five species were grown on cocoons, larvae, and unidentifiable insects. Although a few *Cordyceps* and allied species have been reported from the Western Ghats or west coast regions (Karun & Sridhar, 2013; Kumar & Aparna, 2014; NagRaj, 1962; Nanaware, 2002; Patil, Dangat, & Patil, 2014; Prathibha, 2015), for the first time as many as six morphologically different *Cordyceps* have been reported by Dattaraj, Jagadish, Sridhar, and Ghate (2018). Among the macrofungi found in our study, eight species have dual benefits (edible and medicinal, five species; edible and ectomycorrhizal, two species; medicinal and ectomycorrhizal, one species) (see Table 12.2).

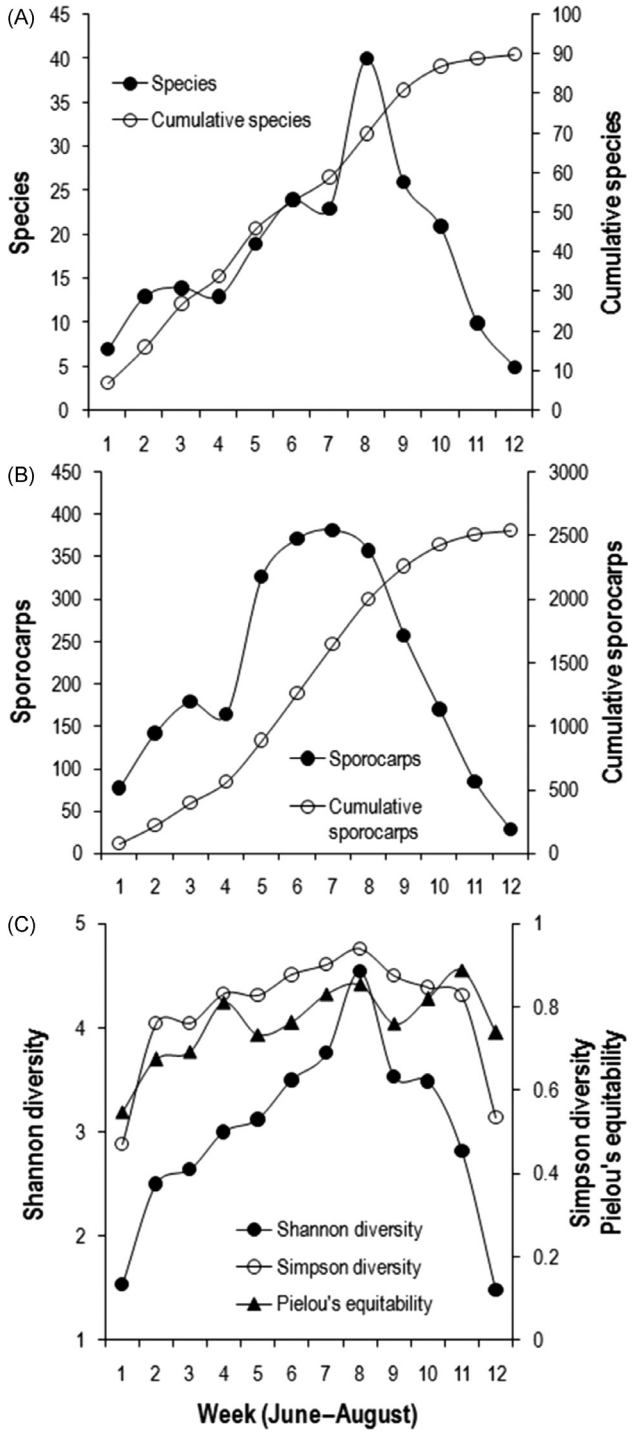

FIGURE 12.2 Species richness and cumulative species (A); sporocarp richness and cumulative sporocarps (B); diversity and equitability (C) of macrofungi over 12 weeks during southwest monsoon.

12.8 Conclusions

The scrub jungles of southwest India provide a variety of substrates and niches for the growth and perpetuation of macrofungi. The present study in scrub jungles over 3 months at weekly intervals showed major differences in the species richness as well as the diversity of macrofungi compared to the earlier surveys. Among the 90 species of macrofungi, up to 50% comprised economically viable fungi with medicinal uses highest, followed by edible and

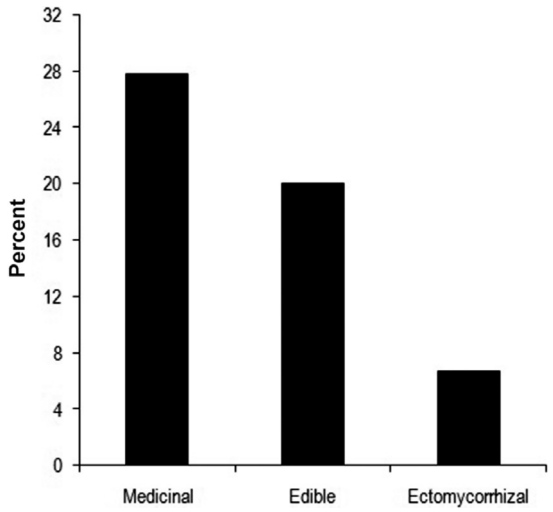

FIGURE 12.3 Percent medicinal, edible, and ectomycorrhizal fungi.

TABLE 12.3 Species richness and diversity in and around scrub jungles of southwest India.

Location	Survey	Species richness	Diversity		Reference
			Simpson	Shannon	
Plantations					
Acacia plantation	Monthly	15	0.854	3.318	Karun and Sridhar (2014)
Areca plantation	Monthly	22	0.877	3.522	Karun and Sridhar (2014)
Cashew plantation	Monthly	17	0.685	2.420	Karun and Sridhar (2014)
Botanical gardens					
Arboretum	Monthly	30	0.751	3.138	Karun and Sridhar (2014)
Arboretum	Biweekly	29	0.885	2.162	Pavithra et al. (2016)
Botanical garden	Biweekly	36	0.892	2.037	Pavithra et al. (2016)
Trees					
Cashew trees	Weekly	22	0.853	3.376	Jagadish et al. (2019)
Terminalia trees	Weekly	36	0.901	3.391	Jagadish et al. (2019)
Scrub jungles					
Scrub jungle	Biweekly	34	0.898	4.129	Greeshma et al. (2016)
Scrub jungle	Weekly	90	0.961	5.281	Present study

ectomycorrhizal species. The occurrence of six species of *Cordyceps* shows the significance of scrub jungles in highly valuable medicinal fungi. Owing to the influence of abiotic factors in scrub jungles (e.g., lateritic soil, near shore, dry−wet regimes, nature of vegetation, temperature, and humidity), the short interval of assessment (e.g., twice or thrice a week) during the southwest monsoon season will provide more significant findings on the assemblage, species richness, and diversity of macrofungi. The present study testifies to the potential of scrub jungles in catering for the needs of economically viable macrofungi, thus warranting intense efforts to conserve this ecosystem for future benefits.

TABLE 12.4 Core group of macrofungi in and around scrub jungles of southwest India.

	Plantations		Scrub jungle	
	Karun and Sridhar (2014)	Pavithra et al. (2016)	Greeshma et al. (2016)	Present study
Astraeus odoratus[a,c]	–	–	+	–
Byssonectria fusispora	+	–	–	–
Chlorophyllum molybdites	–	–	+	–
Collybia aurea	–	+	–	–
Crepidotus uber	+	–	–	–
Cyathus striatus[b]	–	–	+	–
Dacryopinax spathularia[a]	–	–	+	+
Geastrum triplex[b,c]	–	+	+	+
Ileodictyon gracile	+	–	–	–
Lentinus squarrosulus[a,b]	–	–	+	–
Lenzites betulinus[b]	–	–	–	+
Lenzites vespacea	+	–	–	–
Marasmius guyanensis	+	+	–	+
Marasmius haematocephalus	–	–	–	+
Marasmius katangensis	–	–	–	+
Marasmius rotula	–	+	–	+
Marasmius spegazzinii	+	+	–	–
Microporus vernicipes	–	–	–	+
Microporus xanthopus	+	–	–	–
Mycena adscendens	–	–	–	+
Mycena vitilis	+	–	–	–
Phallus indusiatus[a,b]	–	–	+	–
Pycnoporus sanguineus[b]	–	–	+	–
Ramaria pallida	–	+	–	–
Schizophyllum commune[a,b]	–	–	+	–
Tetrapyrgos nigripes	–	+	–	+
Thelephora palmata[c]	–	–	+	–
Trametes versicolor	+	–	–	–
Xylaria hypoxylon[b]	+	–	+	+
Xylaria minuta	–	–	–	+
Xylaria nigripes	+	–	–	–
Xylaria polymorpha[b]	–	–	–	+

[a] *Edible.*
[b] *Medicinal.*
[c] *Ectomycorrhizal.*

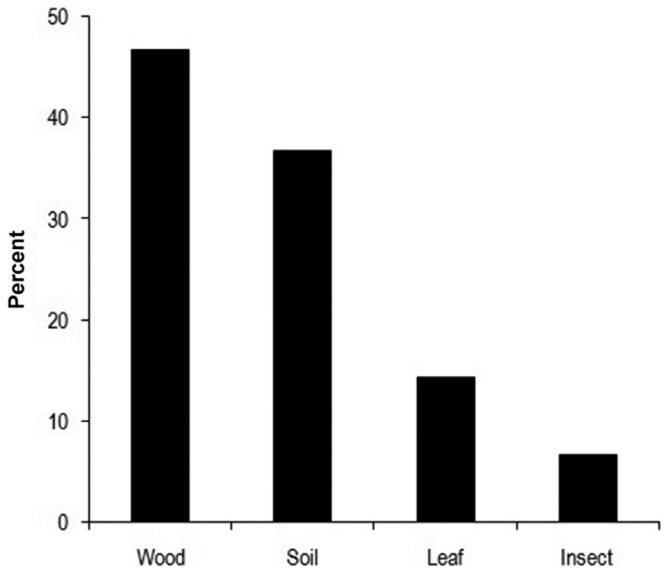

FIGURE 12.4 Percent occurrence of macrofungi on different substrates.

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Chapter 13

Mushroom and plant extracts as potential intervention supplements in diabetes management

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13.1 Introduction

As defined by the World Health Organization (https://www.afro.who.int/health-topics/traditional-medicine), "traditional medicine refers to the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness." In the Occident, where traditional medicine is not part of the healthcare system, traditional medicine is considered as "complementary" or "alternative" (WHO, 2002). Pharmaceutical research has made tremendous strides in the last several decades, with inherited compounds from natural sources serving as a valuable repository of bioactive molecules. In light of this, the preservation of biodiversity is a very important goal within the 17 Sustainable Development Goals set by the United Nations (Neergheen-Bhujun et al., 2017). Knowledge and awareness of ecology and taxonomy is valuable at this time when declining biodiversity of species and their rates of extinction are 100−1000 times higher than in the past (Pimm et al., 2014). Each habitat that has disappeared deprives humans of potential sources of remedies and drugs (Fig. 13.1). Therefore sustainable continuance of natural products is inconceivable without the preservation of biodiversity (Neergheen-Bhujun et al., 2017).

Medicinal mushrooms and plants produce chemical compounds as part of their normal metabolic activities and their use in the prophylaxis or in therapy of different diseases is long-established. Plants and mushrooms provide a rich source of constituents used in drug synthesis and development. Medicinal plants are frequently used for the isolation of biologically active compounds that are used for the preparation of various drugs. Primary metabolites in plants comprise carbohydrates, amino acids, nucleotides, fatty acids, steroids, and lipids, while secondary metabolites in plants encompass terpenoids, nitrogen metabolites, phenolics, saponins, flavonoids, and tannins (Wink, 2015). Some clinically approved medicines, such as laxatives, blood thinners, antibiotics, or antimalarial medications, contain constituents that originate from plants (Wink, 2015). Several mushrooms have been considered as natural health foods as well as remedies in disease therapy (De Silva, Rapior, Hyde, & Bahkali, 2012). Secondary metabolites in mushrooms are polysaccharides, polyphenols, lectins, terpenes, alkaloids, and antibiotics. Medicinal plants and mushrooms possess antioxidant, antiinflammatory, antitumor, antimutagenic, antihepatotoxic, and antidiabetic properties (Kulkarni, Garud, Oza, Barve, & Gaikwad, 2016).

Diabetes, a metabolic disease characterized by disturbed insulin-signaling and resulting hyperglycemia, provides one example of a disease that has been treated in the traditional ways worldwide. There are numerous publications from research groups investigating different plant and mushroom extracts and isolated compounds in the treatment of diabetes and diabetes-related complications.

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FIGURE 13.1 Importance of the preservation of biodiversity. The decline of species and their rates of extinction are 100−1000 times higher nowadays than in the past. Each habitat that has disappeared deprives humans of potential sources of remedies and drugs and therefore sustainable continuance of natural products is inconceivable without the preservation of biodiversity.

13.2 The effect of hyperglycemia in cells

Hyperglycemia is the hallmark of both diabetes types 1 and 2, and it is the main pathophysiological factor in the development of diabetes and its complications. High glucose concentrations in cells lead to increased polyol and hexosamine pathway flux, the activation of protein kinase C (PKC), and increased formation of advanced glycation end products (AGEs) (Brownlee, 2005). AGEs are a diverse group of molecules which are formed in multistep complex reactions. In Maillard's reaction, the carbonyl groups of glucose react with the free amino groups of amino acids, leading to the formation of unstable Schiff bases and more stable Amadori products (Ahmed, 2005). While these reactions are reversible, Schiff bases and Amadori products can react with amino acid residues of tissue or circulating peptides or proteins and result in protein cross-links and the formation of protein adducts in an irreversible process (Ahmed, 2005). Hyperglycemia inside the cell also leads to the reduction of glucose to sorbitol by aldose reductase and subsequent oxidation in the fructose activating polyol pathway flux (Tang, Martin, & Hwa, 2012). Aldose reductase, the rate-limiting enzyme for this pathway, consumes cofactor NADPH, which is important for the regeneration of the intracellular antioxidant, glutathione (Tang et al., 2012). Depletion of NADPH in the activated polyol pathway impairs intracellular antioxidant defenses and increases cellular oxidative stress. Increased cellular glucose concentration promotes synthesis of diacylglycerol known as a cofactor of protein kinase C (PKC) isoforms. The activation of PKC is reflected in gene expression associated with diverse processes, such as cell proliferation and death, extracellular matrix synthesis, regulation of cytokine activity, and increased vascular permeability and contractility (Geraldes & King, 2010). Some of the detrimental effects of hyperglycemia result from the increased hexosamine pathway flux. Under normal circumstances, 2%−5% of the glucose taken by the cells is converted to UDP-N-acetylglucosamine (UDP-GlcNAc) by the hexosamine pathway. Enzymatically regulated addition of the N-acetyl glucosamine (GlcNAc) moiety of UDP-GlcNAc to serine or threonine results in O-GlcNAc modification of proteins (Zachara & Hart, 2004). O-GlcNAcylation is considered to act

analogously to protein phosphorylation. O-GlcNAc modifications strictly correlate with the level of UDP-GlcNAc synthesized through the hexosamine pathway, which is determined by the level of glucose in the cell, and are increased in diabetic conditions (Issad & Kuo, 2008). The increased hexosamine pathway flux was found to mediate many of the adverse effects of hyperglycemia, and unambiguous involvement of O-GlcNAc in cellular processes related to hyperglycemia-induced complications has been documented (Fig. 13.2) (Karunakaran & Jeoung, 2010; Zachara & Hart, 2004).

13.3 Oxidative stress in diabetes

In addition to the formation of AGEs, increased hexosamine and polyol pathway flux, and activation of PKC isoforms, increased production of reactive oxygen species (ROS) occurs in all cells damaged by hyperglycemia. Hyperglycemia- and hyperlipidemia-induced production of ROS and oxidative stress appears to play a pivotal role in the development of diabetes and its complications. In diabetes, the activated electron transport chain in mitochondria also contributes to ROS production (Brownlee, 2001). Hyperglycemia and the subsequent production of ROS impair pancreatic β-cell functions, insulin gene expression, and insulin secretion. In order to neutralize the deleterious effects of free radicals, enzymatic and nonenzymatic components of the antioxidative system (AOS) are activated with the aim of lowering elevated ROS levels and protecting cells from their toxic effects (Pham-Huy, He, & Pham-Huy, 2008). The main enzymatic components of the AOS are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), while the nonenzymatic components include glutathione, ascorbic acid, tocopherol, retinoids, selenium, albumin, transferrin, ceruloplasmin, and uric acid. Oxidative stress also affects epigenetic modifications which are responsible for rapid responses to changes in environmental stimuli (Intine & Sarras, 2012). Moreover, modifications of epigenetic marks also contribute to the ability of cells to "memorize" these encounters and contribute to additional molecular mechanisms that underlie chronic diabetic complications (Intine & Sarras, 2012). Considering the role of oxidative stress in the etiology of diabetes, antioxidant therapy is an important approach for the prevention and treatment of diabetes and its complications.

13.4 Mushroom and plant extracts in the treatment of diabetes

13.4.1 *Centaurium erythraea* Rafn

C. erythraea Rafn (Gentianaceae family), known as small centaury, is a plant species that abundantly grows in Europe and parts of western Asia and northern Africa (Stefkov et al., 2014). It is used as a medicinal herb in numerous Mediterranean countries to treat digestive disorders, febrile conditions, hepatitis, and diabetes (Hatjimanoli & Debelmas, 1977; Jarić et al., 2015; Jouad, Haloui, Rhiouani, El Hilaly, & Eddouks, 2001). Traditional use of *C. erythraea* (CE) in treating diabetes is supported by studies indicating antidiabetic (Hamza et al., 2010), antiinflammatory (Berkan, Ustunes, Lermioglu, & Ozer, 1991; Kumarasamy, Nahar, Cox, Jaspars, & Sarker, 2003), and antioxidant (Valentao et al., 2001) properties of CE extract or its constituents. Animal studies revealed that CE extract lowered

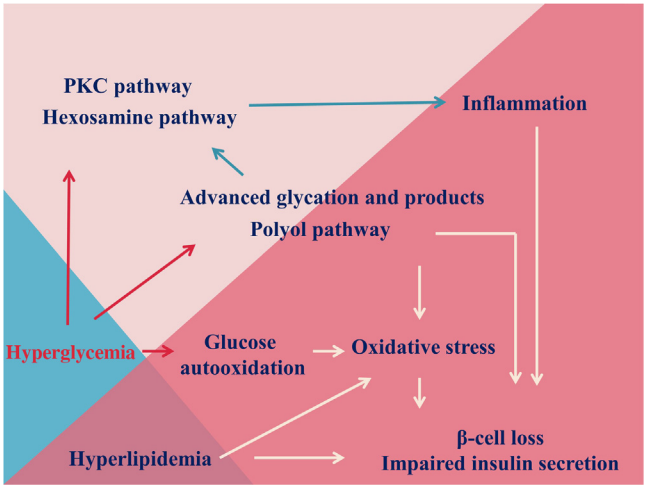

FIGURE 13.2 The effect of hyperglycemia in diabetes. Increased level of glucose in diabetes leads to glucose autooxidation, production of free radicals, and oxidative stress. Hyperglycemia induces activation of polyol, protein kinase C (PCK) and hexosamine pathways, and production of advanced glycation end products. Those activated mechanisms lead to beta cell loss, impaired insulin production, inflammation, and hyperlipidemia.

hyperglycemia and improved serum lipid status in STZ-diabetic rats (Đorđević et al., 2017; Sefi et al., 2011; Stefkov et al., 2014). The influence of CE extract on the regulation of elevated concentrations of blood glucose and carbohydrate-related disturbances was similar to glibenclamide (Stefkov et al., 2014). The hypoglycemic effect of CE extract could be ascribed to its effect on the stimulation of glycogen synthesis and reduction of gluconeogenesis in the liver. The authors suggested that bitter compounds such as secoiridoids and xanthones that were identified as predominant components of CE extract could also contribute to normalization of glucose levels and lipid metabolism by stimulating excretion in the gastrointestinal tract, including increased secretion of insulin from the remnant pancreatic beta cells. Treatment with CE extract increased insulin levels in the circulation of diabetic animals, partially as the result of CE extract-mediated protection of pancreatic islets from oxidative stress-induced damage (Sefi et al., 2011). Đorđević et al. (2017) have shown that CE extract protected erythrocytes from damage, probably through nitrogen oxide and hydrogen peroxide-scavenging activities and by reduction of protein glycosylation and glycation associated with hyperglycemia (Đorđević et al., 2017). By increasing prosurvival Akt kinase activity in red blood cells and by reducing serum alpha-2-macroglobulin levels, CE extract improved the functional properties of erythrocytes and consequently microcirculation in diabetes, thus contributing to the enhanced oxygen supply to tissues and organs. Valentao et al. (2001) have demonstrated the in vitro ability of a CE infusion to scavenge hydroxyl radicals, superoxide anions, and hypochlorous acid (Valentao et al., 2001). The antioxidant properties of the CE extract could be ascribed to the polyphenols detected in the CE extract, such as apigenin, luteolin, quercetin, astragalin, isoquercitrin, naringenin, caffeic, sinapic, ferulic and p-coumaric acids, and xanthones (Đorđević et al., 2017; Stefkov et al., 2014). In addition, secoiridoid swertiamarin, the prevalent component of the CE extract, stimulated activity of antioxidant enzymes in the serum, liver, and kidneys and restored antioxidant mechanisms in the experimental model of acute liver damage in rats (Jaishree & Badami, 2010). The CE extract displayed a hepatoprotective effect in type 2 diabetic mice, being thus a promising candidate for prevention or treatment of nonalcoholic liver steatosis (Hamza et al., 2015). Taken together, further elucidation of the effects of CE extract and its components provides a promising platform for the development of novel medicaments to treat diabetes and its complications (Fig. 13.3).

13.4.2 *Castanea sativa*

C. sativa (sweet chestnut) is a species from the Fagaceae family that can be found in the Mediterranean region of Europe and in some parts of Asia. *C. sativa* (CS) is known as a valuable source of bioactive, mainly polyphenolic compounds which are responsible for the majority of the observed beneficial antiinflammatory (Schink et al., 2018), antiviral (Lupini, Cecchinato, Scagliarini, Graziani, & Catelli, 2009), and antioxidant effects (Mujić et al., 2011). In the last

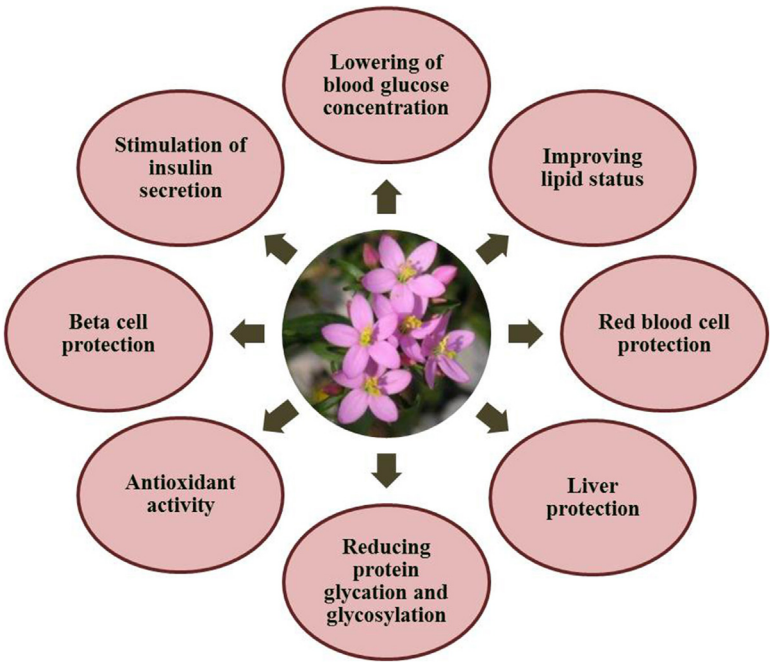

FIGURE 13.3 An overview of the protective effects of *C. erythraea* in the treatment of diabetes. Shown are different activities of small centaury extract that contribute to alleviating the symptoms of diabetes.

decade, several papers have suggested the potential use of CS extracts in the treatment of pathophysiological conditions related to oxidative stress and inflammation as occur in diabetes mellitus. The antioxidant activity of extracts derived from various parts of *C. sativa* has been a subject of several in vitro studies. Cardullo, Muccilli, Saletti, Giovando, and Tringali (2018) examined the antioxidant and hypoglycemic activities of the ethanolic extract of the commercially available tannin obtained from CS (Cardullo et al., 2018). Fractionation and subsequent analysis identified several constituents of polyphenolic fractions, previously reported as α-glucosidase inhibitors, with high antioxidant and hypoglycemic activities (Cardullo et al., 2018). Comparison of the antioxidant capacity of the ethanolic extracts derived from seed, peeled seed, outer brown peel, leaf, and catkin of CS revealed that extracts obtained from the leaves, catkin, and outer brown peel possessed high antioxidant activity in scavenging hydroxyl and DPPH radicals, which was in correlation with the total content of phenolics (Živković et al., 2009). In line with in vitro tests, in cellulo experiments showed that extracts from CS leaf, catkin, and spiny burrs protect β-cells from cell death induced by the commonly used experimental diabetogenic agent streptozotocin (STZ) (Mujić et al., 2011). The authors showed that CS extracts protected DNA and lipids from oxidative damage and improved the redox status by enhancing the endogenous antioxidant system and increasing the concentrations of reduced glutathione. It has been suggested that extract of CS spiny burrs possesses significant antioxidant potential based on its reducing power, chelating effects, and radical-scavenging activity (Grdović et al., 2012). The antioxidant capacity of the extract of spiny burrs corresponded to the high content of phenolics and flavonoids, identified by HPLC analysis as predominantly being ellagic acid, gallic acid, and their derivatives, and flavonoid compounds. The chestnut extract not only increased β-cell viability after STZ treatment, but also preserved the functioning of β-cells, based on the amount of secreted insulin (Grdović et al., 2012). The antidiabetic effects of the CS extract derived from spiny burrs were evaluated in vivo on hepatorenal injury in STZ-induced diabetic rats (Arambašić Jovanović et al., 2017). The extract improved the glycemic and lipid status, the redox status, and reduced oxidative damage of DNA and lipids. Moreover, the CS extract showed strong antiglycation activity both in vitro and in vivo. The extract-induced decrease in nonenzymatic glycosylation was accompanied by inhibition of RAGE/NF-κB activity, as well as by the diminishment of O-GlcNAcylation in liver and kidney of diabetic rats (Arambašić Jovanović et al., 2017). The in vivo antioxidant and antigenotoxic potential of CS wood extract was also revealed in polyunsaturated fatty acid (PUFA)-induced oxidative stress in pigs (Frankic & Salobir, 2011). A recent study found that CS bark extract, which is rich in ellagitannins, protects the liver and intestine in high fat diet (HFD)-overweight rats (Budriesi et al., 2018). The authors suggested that the chestnut bark extract is a promising nutraceutical tool as it decreased spontaneous food consumption, restored liver phase I and II enzyme activities, reduced intestinal oxidative stress, and restored intestinal contractility. Aside from its antiadipose and antioxidative activities, the CS bark extract was also shown to possess antiinflammatory activity as it lowered the level of proinflammatory mediators and restored the levels of antiinflammatory cytokines (Budriesi et al., 2018). In agreement with this observation are the recently documented antiinflammatory effects of CS leaf extract (Schink et al., 2018). Strong antiinflammatory effects of the chestnut leaf extract, manifested as decreased IL-8 concentrations and potent TLR2 and TLR4 antagonistic activities, point to the CS leaf extract as a potentially interesting novel strategy in treating inflammatory diseases. Taken together, current data strongly advocate further studies of the effects of CS extracts in the development and progression of diabetes. Beside the proven antioxidative effects, antiinflammatory activity has emerged as an important intrinsic feature of chestnut extracts. As diabetes represents a chronic inflammatory condition accompanied by oxidative stress, treatment with CS extract could provide a desirable strategy for attenuating diabetes and its complications (Fig. 13.4).

13.4.3 β-Glucan-enriched cereal grain extracts

Beta-glucans (BGs) comprise a group of β-D-glucose polysaccharides from cell walls of cereals, fungi, yeast, and bacteria (Andrade & Orlando, 2018). BG isolated from cereals or grass contains a central linear β-type (1−3) and branching hydrocarbon chain with β-bonds (1−4), while the BGs of fungi and yeasts have β-bonds (1−6). BGs are a widely examined class of plant-derived polysaccharides and a variety of their biological activities have been revealed. New studies have assigned BGs to functional foods as prebiotics possessing immunomodulatory activities, antiinfective and antitumorigenic properties, with effects on carbohydrate and lipid metabolism (Brown & Gordon, 2003; De Natale et al., 2012; Vetvicka, 2011). Dietary intake of BGs has been shown to lower hyperglycemia, hyperlipidemia, and hypertension, pointing to the beneficial role of BGs in the treatment of diabetes and diabetes-associated complications (Rahar, Swami, Nagpal, Nagpal, & Singh, 2011). Lo, Tsai, Wasser, Yang, and Huang (2006) showed on the experimental model of streptozoticin-induced diabetes in rats that glucans from fungi and oats attenuated blood glucose concentrations, pointing to their potential role as an oral hypoglycemic agent and functional food for diabetes management (Lo et al., 2006). The underlying mechanism through which glucans lower glucose concentration is related to the formation of a

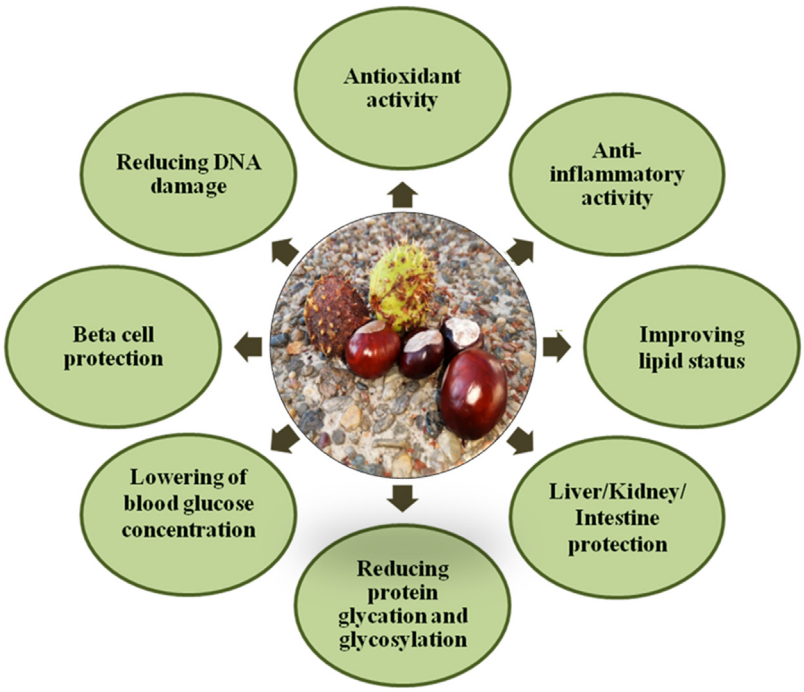

FIGURE 13.4 Schematic representation of the protective effects of *Castanea sativa* in diabetes management. Beneficial effects of *C. sativa* extracts derived from leaves, wood, bark, catkin, and spiny burrs in attenuation of diabetes and the complications are summarized.

gelatinous layer in the gut which delays the absorption of glucose and lipids (Liatis et al., 2009; Tappy, Gugolz, & Wursch, 1996). The BG-enriched extract from cereal grains in in vitro experiments exhibited significant concentration-dependent free radical, hydrogen peroxide, and nitric oxide scavenging activities, as well as metal chelating activity (Mihailović et al., 2013a, 2013b). In in vivo experiments on streptozotocin-induced diabetes in rats, application of a BG-enriched extract from cereal grains attenuated hepatic oxidative stress by decreasing the level of thiobarbituric acid reactive substances and by restoring the ratio of reduced glutathione to oxidized glutathione (Uskoković et al., 2013). In addition, a BG-enriched extract from cereal grains exhibited an effect on the systemic regulation of redox disturbance, contributing to reduced DNA damage in the liver and kidney of diabetic rats (Mihailović et al., 2013a, 2013b). The mechanism underlying the beneficial effect of the BG-enriched extract from cereal grains in diabetic rats involved the activation of the prosurvival pathways through Akt kinase activation and diminished degradation of procaspase-3 (Mihailović et al., 2013a, 2013b). Treatment of diabetic rats with the BG-enriched extract from cereal grains resulted in significantly reduced serum protein glycation, decreased formation of advanced glycation end products (AGE) in vitro and in vivo, displayed as lower levels of O-GlcNAc-modified superoxide dismutases and catalase in liver and kidney (Mihailović et al., 2013a, 2013b). The BG-enriched extract from cereal grains also exerted a hepatic antiinflammatory effect in diabetic rats by stimulation of IL-4 and IL-10 antiinflammatory cytokine mRNA expression, inhibition of RAGE/NF-κB signaling, and restoration of the serum levels of the acute-phase protein α2-macroglobulin, and albumin (Uskoković et al., 2013). These results suggest that administration of the BG-enriched extract from cereal grains under diabetic conditions promoted beneficial antihyperglycemic, antioxidant, and antiinflammatory effects, which increased liver and kidney resistance to the onset of diabetic complications (Fig. 13.5).

13.4.4 *Lactarius deterrimus*

Mushrooms and their extracts have been used in traditional medicine for thousands of years as foods to maintain good health and as therapeutic agents to treat disease in all parts of the world. Due to the fact that mushrooms have high contents of proteins, vitamins, and minerals and are deficient in simple carbohydrates, saturated fats, and cholesterol they are a perfect food for diabetic patients (De Silva et al., 2012; Lo & Wasser, 2011). Besides well-known medicinal mushrooms that possess hypoglycemic and antidiabetic effects (*Ganoderma lucidum*, *Inonotus obliquus*, *Phellinus linteus*, *Cordyceps sinensis*, *Coprinus comatus*, *Agaricus blazei*, *Agaricus bisporus*, *Poria cocos*, *Sparassis crispa, Grifola frondosa*, etc.) (De Silva et al., 2012), *Lactarius deterrimus* is an edible mushroom that also demonstrates antidiabetic properties and therefore is important in the management of diabetes (Mihailović et al., 2015). *L. deterrimus*, also known

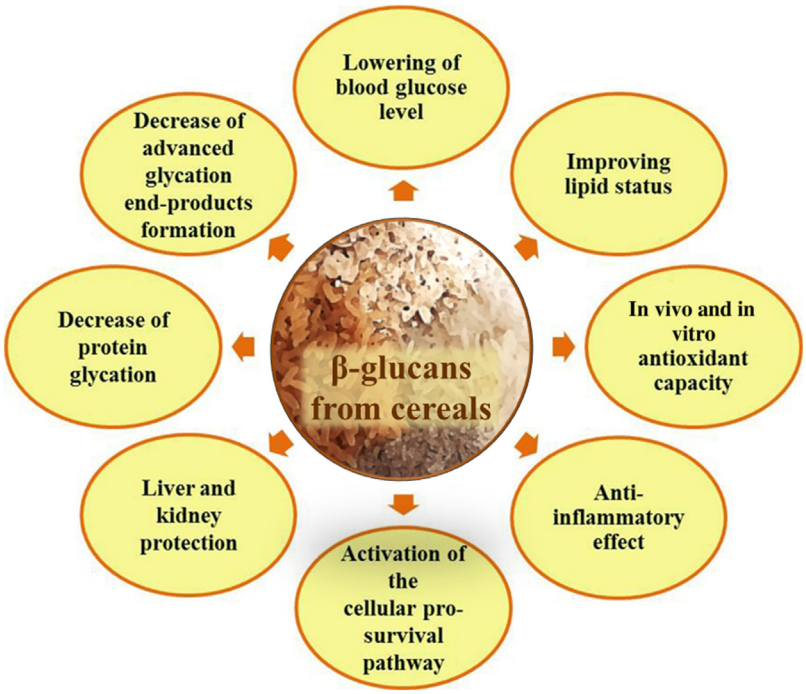

FIGURE 13.5 Ameliorating effects of beta-glucans from cereals in management of diabetes and diabetic complications. Presented are different activities of beta-glucans involved in improving diabetic condition and contributing to slowing down the development of diabetic complications.

as false saffron milkcap or orange milkcap, belongs to the family Russulaceae (class *Basidiomycotina*) and is distributed mainly in coniferous woods in northern, northeastern, and Central Europe. In addition to antidiabetic effects, *L. deterrimus* and other *Lactarius* species, such as *L. salmonicolor*, *L. deliciosus*, and *L. sanguifluus*, exhibit also antitumor, antioxidant, antimicrobial, and immunostimulatory actions (Athanasakis, Aligiannis, Gonou-Zagou, Skaltsounis, & Fokialakis, 2013; Hou et al., 2013). Reported antioxidant properties of *L. deterrimus* extract represent a promising potential for attenuation of oxidative stress-related processes included in the initiation and progression of diabetes and diabetes-induced complications. Two independent studies analyzed antioxidant activities of *L. deterrimus* extract in different test systems and showed relatively low radical-scavenging activity in the DPPH assay, moderate reducing power, and good nitrogen oxide- and hydrogen peroxide-scavenging activities (Grdović et al., 2012; Sarikurkcu, Tepe, & Yamac, 2008). These antioxidant properties of extracts were in correlation with the reported content of phenolics and flavonoids in the ethanolic extract of *L. deterrimus* (Grdović et al., 2012). Also, *L. deterrimus* showed a potential to inhibit glycation of proteins and formation of AGEs in vitro (Arambašić Jovanović et al., 2017), processes which are accelerated in diabetes and lead to hyperglycemia-induced complications. These results were further verified in vivo on a rat model of STZ-induced diabetes where the administration of the *L. deterrimus* extract to diabetic rats improved hyperglycemia, reduced glycated hemoglobin, glycated serum protein, and AGE levels, increased activities of antioxidant enzymes, SOD, and CAT in the circulation, and increased levels of free intracellular thiols and glutathionylated proteins (Mihailović et al., 2015). In the study of Grdović et al. (2012) *L. deterrimus* extract exerted protective effects against STZ-induced pancreatic β-cell death through improved oxidative status, prevention of lipid peroxidation and DNA damage, which were explained by strong nitrogen oxide- and hydrogen peroxide-scavenging activity of *L. deterrimus* extract (Grdović et al., 2012). Also, *L. deterrimus* extract exerted in vivo beneficial effect reflected on islet cell proliferation and regeneration through the stimulation of Akt protein kinase prosurvival pathway which led to an increased number of proliferating cell nuclear antigen (PCNA)-positive pancreatic cells (Mihailović et al., 2015). In spite of the weak antihyperglycaemic activity of the *L. deterrimus* extract, Arambašić Jovanović et al. reported the improvement of the hepatorenal function and structure in diabetic rats treated with *L. deterrimus* extract (Arambašić Jovanović et al., 2017). This improvement was correlated with restoration of the antioxidant defense systems and reduction of the nonenzymatic and enzymatic glycosylation in liver and kidney of *L. deterrimus* treated diabetic rats. The reduction of nonenzymatic glycosylation interrupted the detrimental CML-mediated RAGE/NF-κB activation linked to a pronounced tissue injury. Also, by limiting the level of O-GlcNAcylation, it has been suggested that *L. deterrimus* extract changes the performance of specific proteins and preserves normal cell signaling pathways that are disturbed in the liver and kidney of diabetic rat. These findings suggest that *L. deterrimus* extract has a positive impact on different

FIGURE 13.6 Protective effects of *Lactarius deterrimus* in the treatment of diabetes. Review of the positive effects of *L. deterrimus* extract on different processes involved in the onset and progression of diabetes and its complications.

harmful cellular processes involved in the onset and progression of diabetes and diabetic complications and because of that needs to be further evaluated for its effectiveness and safety in clinical studies (Fig. 13.6).

Acknowledgments

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Chapter 14

Anticancer activities of marine macroalgae: status and future perspectives

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14.1 Introduction

Cancer is a global crisis, causing significant threat to human health and life (Plummer et al., 2016). Chemotherapy is the backbone of treatment for many cancers; however, the emergence of chemotherapeutic resistance has led to reduced therapeutic success. This has resulted in efforts being directed toward the development of new drugs and forms of chemotherapy (Alfarouk et al., 2015; Links & Brown, 1999). The search for natural drugs to inhibit or cure cancer has been ongoing for more than four decades, in the hope of finding less hazardous cancer treatments. In this quest, marine flora and fauna have been considerably studied. Marine organisms are from a wide range of evolutionary origins, comprising many marine taxa that do not have terrestrial equivalents. With more than 28,000 molecules isolated from marine organisms (Blunt, Copp, Keyzers, Munro, & Prinsep, 2015; Jaspars et al., 2016), many of them have proven bioactivity (Vignesh, Raja, & James, 2011). Five approved marine drugs are already available in the market, while three others are in anticancer clinical phase III trials (Malve, 2016). Consideration of molecules and compounds from marine organisms for the exploration of anticancer properties led to the discovery of numerous promising drugs or compounds with marketing potential (Cragg, Grothaus, & Newman, 2009).

For the past decades, promising anticancer activities from marine invertebrates were emphasized, and have yielded highly cytotoxic compounds that have successfully completed clinical trials and been patented as anticancer drugs (Bhatnagar & Kim, 2010). However, during recent years there have been an upsurge of studies on anticancer properties more focused on marine macroalgae. This has involved a search for less toxic compounds, as a variety of elements isolated from marine invertebrates were highly toxic to normal tissues during preclinical trials (Amador, Jimeno, Paz-Ares, Cortes-Funes, & Hidalgo, 2003; Mayer et al., 2010). There is also evidence that the long history of use of marine macroalgae in diets has resulted in lower rates of noncommunicable diseases (NCDs) (Yuan & Walsh, 2006). Being edible, many marine macroalgae are expected to produce lower toxicity to normal tissues. Marine macroalgae became recognized as sources of bioactive chemicals, including sulfated polysaccharides and polyphenols (Le Tutour et al., 1998). Macroalgae are organisms that carry out chloroxygenic photosynthesis, do not belong to the group Embryophyta (Bolton, 2016), and are visible to the naked eye. They belong to well-known phyla that are Chlorophyta (green seaweeds), Phaeophyceae, Ochrophyta (brown seaweeds), and Rhodophyta (red seaweeds) (Diaz-Pulido and McCook, 2008).

Direct effects of secondary metabolites or biosynthetically derived compounds from marine macroalgal species are being explored for anticancer properties. In this line, there is a need for a review of recent studies of anticancer effects of molecules and extracts derived from macroalgae, to facilitate faster and less expensive screening procedures for a subsequent exploration of these organisms for their anticancer properties. Moreover, a detailed discussion of cell death mechanism, antiangiogenic, antiinvasive, and antimetastatic activities, as well as their molecular action mechanisms

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TABLE 14.1 Cytotoxic, antiproliferative, or growth inhibitory activities of compounds isolated from marine macroalgae.

Compounds	Type	Species	Cell lines	Inhibition/$1C_{50}$/GI_{50}	References
Alginates	Brown seaweed	*Sargassum vulgare*	Sarcoma 180 cells in mice	74.2%−88.8% inhibition	De Sousa et al. (2007)
Comosusol A-D		*Sporochnus comosus*	MCF-7 (human breast adenocarcinoma), SF-268, H460, CHO-K1 and HT-29 cells	GI_{50} ranged between 5 and 67 μM against	Ovenden et al. (2011)
Fucoidan		*Undaria pinnatifida*	PC-3, HeLa, A549, and HepG2	IC_{50} ranged between 0.1 and 0.8 mg/ml	Synytsya et al. (2010)
Fucoidan		*Padina* sp., *Fucus vesiculosus*	MCF-7, WiDr and Vero	IC_{50} of 144, 118 and 501 μg/mlL MCF, WiDr and Vero cells, respectively. Standard fucoidan from *Fucus vesiculosus* exhibited IC 50 of 60, 63, and 211 μg/ml against MCF-7, WiDr and Vero cells, respectively.	Isnansetyo, Lutfia, Nursid, Trijoko & Susidarti (2017)
Fucosterol		*Sargassum* spp.	T47D and HT-29	IC_{50} 82.26−190.24 μg ml	Khanavi et al. (2012)
Meroditerpenoids		*Sargassum* spp.	Hela	0.1−2 mg/ml of compound enhanced growth inhibition	Pereira et al. (2011)
Stypolactone		*Stypopodium zonale*	HT-29, H-116 and A549	$IC_{50} < 2.5$ μg/ml	Dorta, Cueto, Diaz-Marreto & Drias (2002)
Bromophycoic acids A-E	Red seaweed	*Callophycus* sp.	14 cancer cell lines	IC_{50} ranged between 6.8 and 36 μM	Teasdale et al. (2012)
Cuparene sesquiterpenes		*Laurencia microcladia*	a549 and NSCLC-N6	IC_{50} ranged between 52.4 and 242.8 μM against a549 cells and 73.4−193.9 μM against NSCLC-N6 cell line	Kladi et al. (2005)
Halogenated monoterpene aldehydes		*Plocamium corallorhiza*	WHCO1 esophageal cancer cells	IC_{50} was 6.6−9.9 μM	Antunes et al. (2011)
Halomon		*Portieria hornemannii*	60 cell lines	GI_{50} ranged between 0.6 and 33 μM	Fuller et al. (1994)
Laurenditerpenol		*Laurencia intricata*	Breast tumor cells	Inhibited HIF-1 mediated hypoxic signaling	Mohammed et al. (2004)
Laurinterol		*Laurencia okamurae*	Melanoma cells	Cytotoxicity IC_{50} at 1−50 μg/ml	Kim, Mendes, & Kim (2008)
Lophocladine		*Lophocladia sp.*	NCI-H460 and MDA-MB-435 cells	Were Cytotoxic at 64.6 μM and 3.1 μM, respectively	Gross et al. (2006)
Prevezols B-E		*Laurencia obtusa*	5 Human cell lines	IC_{50} ranged between 34 and 200 μM	Iliopoulou, Mihopoulos, Vagias, Papazafiri & Roussis (2003)
Thyresenol A and B		*Laurencia tristicha*	ZR-75-1, T47D, Hs578T and MDA-MB-231 cells	IC_{50} range between 7.9 and 14.8 μg/ml	Pec et al. (2003)

TABLE 14.2 Mechanisms of apoptosis induced by "compounds" of brown macroalgae in cancer cells.

Treatment	Species	Targets	Activity	Mechanism	References
Fucoidan	*Cladosiphon okamuranus*	HTLV-1-infected T-cells Leukemia cells (in vitro) and in mice (in vivo)	Antiproliferative	Downregulation of protein-2, survivin, cyclin D2, c-myc, and hyperphosphorylated form of the retinoblastoma tumor suppressor protein, induction of G1 phase accumulation, inactivation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), activator protein-1 (AP-1), and NF-kB inducible chemokine production and prevented homotypic cell to cell adhesion	Haneji et al. (2005)
Fucoidan	*Fucus* sp.	4T1 cells (in vitro) and in BALB/c mice (in vivo)	Cytotoxicity	Downregulation of vascular endothelial growth factor (VEGF), decreased expression of B-cell lymphoma 2 (Bcl-2) and the ratio of Bcl-2 to Bax, decreased expression of survivin and phosphorylated extracellular signal regulated protein kinases (ERKs), release of cytochrome C, increase in cleaved caspase-3 protein	Xue et al. (2012)
Fucoidan	*Saccharina cichorioides*	HCT 116 cell lines	Cytotoxicity	Activation of initiator caspase-9 and effector caspase-7 and -3, followed by the cleavage of poly ADP ribose polymerase (PARP)	Vischuk, Ermakova, & Zvyagintseva (2013)
Fucoidan	*Fucus vecículosus*	Human lymphoma HS-sultan cells	Antiproliferative	Phosphorylation of ERK and decrease of G protein-coupled receptor kinase (GRK)	Aisa et al. (2005)
Fucoidan	*Fucus vecículosus*	A549 and CL1-5 cells and LLC1-xenograft male C57BL/6 mice	Prevention of tumorigenesis and reduction of tumor size	Induced ER stress response through activation of the PERK-ATF4-CHOP pathway, resulting in apoptotic in vitro and in vivo. Intracellular increased followed by increase of ATF4 and CHOP in lung cancer cells. Using the ROS scavenger N-acetyl-l-cysteine (NAC), involvement of ROS generation in fucoidan-induced ER stress-mediated apoptosis was confirmed. Moreover, via toll-like receptor 4 (TLR4) knockdown, fucoidan-induced attenuation of ROS and CHOP expression was demonstrated.	(Hou et al. (2017)
Fucoidan along with cisplatin, tamoxifen or paclitaxel	*Cladosiphon novae-caledoniae*	MCF-7 and MDA-MB-231 cell lines	Antiproliferative	Downregulation of the antiapoptotic proteins B-cell lymphoma- extra-large (Bcl-xL) and Mcl-1 and a decrease in the phosphorylation of ERK and protein kinase B (Akt) in MDA-MB-231 cells, but increased the phosphorylation of ERK in MCF-7 cells	Jaramillo and Zhang (2013)
Fucoxanthin	*Undaria pinnatifida*	Caco-2 Colon cancer cells	Cytotoxic	Deoxyribonucleic acid (DNA) fragmentation, suppression of Bcl2, DNA fragmentation inhibited in presence of caspase inhibitor, activation receptor (PPAR) gamma	Hosokawa et al. (2004)

(*Continued*)

TABLE 14.2 (Continued)

Treatment	Species	Targets	Activity	Mechanism	References
Heterofucan SF-1.5 v	*Fucus vesiculosus and Sargassum henslowianum*	B16 mouse melanoma cells	Antiproliferative	Loss of plasma membrane asymmetry, translocation of cell membrane phospholipids and activation of caspase-3 observed	Ale et al. (2011)
Sulfated homo-heterofucans	*Sargassum filipendula*	HeLa cell	Antiproliferative	Decrease in expression of Bcl-2 and increased Bax and mitochondrial release of apoptosis-inducing factor (AIF) into cytosol	Costa et al. (2011)

related to macroalgal compounds and molecules are incomplete in current literature, thus preventing a holistic understanding of the molecular processes underlying anticancer effects of various macroalgal species. Reviews describing indirect anticancer activities that inhibit cancer by targeting cellular physiological processes by macroalgal extracts are unavailable. Similarly, few authors have elaborated on the effect of varying environmental conditions on levels of compounds and molecules of marine macroalgae with anticancer properties.

This review thus aims to provide a relevant update on the status of investigations of anticancer properties of marine macroalgae and their derived compounds. It also describes the molecular mechanisms employed by selected macroalgal molecules and compounds. In addition, the effects of varying environmental conditions on the levels of compounds and the resulting anticancer effects of these marine macroalgae will be discussed.

14.2 Status of reported anticancer activities of marine macroalgae

Recent studies have unraveled the anticancer potential of macroalgal species (Tables 14.1–14.5). Of more than 8900 estimated marine algal species (Kilinc, Cirik, Turan, Tekogul, & Koru, 2013), less than 1.5%, including 1.4% of red macroalgae, 2% of green macroalgae, and 6% of brown macroalgae, have been reported for their anticancer effects. With more than 98% of macroalgal species still unexploited, the quest for finding anticancer agents from macroalgal species looks strongly promising. Interestingly, findings reporting in vitro and in vivo anticancer activities of marine macroalgal species have increased considerably over the past two decades. More recently, trials using the brown macroalgal sulfated polysaccharide fucoidan were emphasized (Fig. 14.1A). Most articles reported anticancer activities of brown seaweeds, and red seaweed species (Fig. 14.1B). Compounds like fucoidan secondarily ingested by marine animals, are among the few macroalgal-related compounds to have been tested through clinical trials. Factors including financial, supply, and technical limitations (Martins, Vieira, Gaspar, & Santos, 2014) might have emerged during in-depth assessment of other compounds.

Red macroalgae (El Gamal, 2010) and brown macroalgae (Wijesekara, Pangestuti, & Kim, 2011) have been recognized to have bioactive properties, including anticancer potential. Activities reported in previous reviews were limited to cytotoxic and antiproliferative effects and apoptosis inducing cell death (Bhatnagar & Kim, 2010; El Gamal, 2010; Moussavou et al., 2014; Pangestuti & Kim, 2011). However, for the past 15 years, anticancer activities of additional macroalgal species including those of green macroalgae, have been described with regards to cytotoxic, antiproliferative activities, transformation inhibition, anti-angiogenic, antiinvasive, antimetastatic activities, and indirect anticancer activities (Fig. 14.2). Interestingly, fucoidans found in various brown seaweed species belonging to the brown macroalga orders Laminariales and Fucales have displayed significant propensities. The applicability of in vivo and in vitro results in the development of fucoidans for use as marine anticancer drugs has also been discussed (Berteau & Mulloy, 2003; Kwak, 2014).

14.3 Cytotoxic, antiproliferative, and growth inhibitory activities of marine macroalgae

Extracts, fractions, and compounds derived from marine macroalgae have yielded promising antitumor actions including cytotoxic, antiproliferative, and growth inhibitory properties against cancer cells in both in vitro and in vivo models

TABLE 14.3 Mechanism of apoptosis induced by "extracts and fractions" of macroalgae in cancer cells.

Treatment	Species	Type	Targets	Activity	Mechanism	References
Polyphenolic fraction	*Ecklonia cava*	Brown	CT-26 cell line	Antiproliferative	Sub-G1 phase arrest, DNA fragmentation, regulation of caspase-7 and -8	Athukorala, Kim & Jeon (2006)
Ethanolic extract	*Gracilaria edulis*	Red	EAT cells	Cytotoxicity	Increased caspase-2, caspase-3 and caspase-9 activities were observed	Patra and Muthuraman (2013)
Ethyl acetate extract	*Hydroclathrus clathratus*	Brown	HL60	Cytotoxicity	caspase 3 and 9 activation, PARP degradation, DNA fragmentation and sub G1 arrest, reduction in level of Bcl-xL, increase levels of Bax proteins, upregulation of Bax/Bcl-2 ratio and reactive oxygen species (ROS) generation.	Kim et al. (2012)
Glyoprotein	*Capsosiphon fulvescens*	Brown	AGS cells	Antiproliferative	Caspase-cascade and PARP activation, release of cytochrome C and apoptotic protease activating factor-1 (APAF-1) from mitochondria to the cytosol. Sub-G1 phase arrest, decrease in the expression of cyclin D, cyclin E, cyclin dependent kinase 2 (Cdk2), Cdk4, and Cdk6, and an increase in the protein levels of p21 and p27	Kim et al. (2012)
Methanolic extract	*Sargassum muticum*	Brown	MCF-7 and MDA-MB-231 cell lines	Antiproliferative	Accumulation of extract-treated cells at sub-G1 phase	Namvar et al. (2013)
Methanolic extract	*Padina pavonia*	Brown	HeLa andMDA-MB-453 cell lines	Cytotoxicity	Increase in cells containing sub-G1 amounts of DNA	Stanojkovic et al. (2013)
Methanolic extract	*Pylaiella littoralis*	Green	HT-29 cells	Antiproliferative	DNA fragmentation observed, decrease Bcl-2 protein and increased Bax expression, activation of caspase-3 and PARP expression via the caspase pathway, increase in phosphorylation of c-Jun N-terminal kinases (JNK), p38, ERK expression via mitogen −activated protein kinases (MAPKs) pathway	Ye et al. (2013)
Methanolic extract	*Chondrus crispus and Palmaria palmata*	Red	Hela cells	Antiproliferative	Induction of caspase 3/7 activity	Athukorala et al. (2016)
Methanolic extracts	*Plocamium telfairiae*	Red	HT-29 colon cancer cells	Cytotoxicity	Regulation of caspases	Kim et al. (2013)

(*Continued*)

TABLE 14.3 (Continued)

Treatment	Species	Type	Targets	Activity	Mechanism	References
Polysaccharide extract	*Capsosiphon fulvescens*	Brown	AGS cells	Cytotoxic	Increase in caspase-3 activation and a decrease in Bcl-2 expression. Decrease in insulin-like growth factor receptor (IGF-IR) signaling and the PI3K/Akt pathway activation	Kwon and Nam (2007)
Polysaccharides fraction	*Hydroclathrus clathratus*	Brown	HL-60 and MCF-7 cell and sarcoma 180 bearing mice	Cytotoxicity and antiproliferative	Sub-G1 arrest observed in vitro. Growth inhibition in vivo along with increased tumor necrosis factor alpha (TNF α) observed	Wang et al. (2010)

TABLE 14.4 Antiangiogenic activity from extract/fraction/compounds of marine macroalgal (mostly fucoidan from brown seaweeds) species.

Molecules	Species	Type	Target	Activities exhibited	References
Polysaccharide	*Sargassum stenophyllum*	Brown seaweed	Chick embryos	Reversing stimulation of basic fibroblast growth factor-stimulated vasculogenesis and decreased the percentage cephalic length of chick embryos	Dias et al. (2008)
Fucoidan	*Undaria pinnatifida*		Human umbilical vein endothelial cells (HUVEC)	Decreased expression of VEGF-A factor	Liu et al. (2012)
Fucoidan	-		Retinal pigment epithelium (RPE) cells and RPE choroid explants	Decreased expression of VEGF-A factor	Dithmer et al. (2014)
Fucoidan	*Laminaria* spp., *Fucus* spp.		MDA-MB-231	Inhibition of human umbilical vein endothelial cells (HUVEC) tubulogenesis	Cumashi et al. (2007)
Fucoidan	–		DU-145 cells in mice	Reduction of phosphorylated JAK and STAT3 in tumor tissue and reduction of VEGF, Bcl-xL and Cyclin D1	Rui et al. (2017)
Fucoidan	*Cladosiphon novae-caledoniae*		HT1080 cells	Suppression of MMP-2/9 expression	Ye et al. (2005)
Sulfated galactan	*Codium cylindricum*	Green seaweed	Serum-free matrix culture model using rat aortic ring	Suppression of microvessel formation and inhibition of human umbilical vein endothelial cells HUVEC tube formation	Matsubara et al. (2005)
Caulerpin	*Caulerpa* spp.		T47D cell	Inhibition of hypoxia-induced and 1, 10-phenanthroline-induced activation of HIF-1; blocking of HIF-α protein in hypoxic condition. Inhibition of NADH-ubiquinone oxidoreductase, inhibiting the transport or delivery of electrons to complex III	Liu et al. (2009a)
Methanolic extract	*Ulva rigida*		Tumor in mice induced by EAC cells (in vivo)	VEGF was maximally reduced	Salem and Ibrahim (2011)
Polysaccharides	*Grateloupia longifolia*	Red seaweed	Sarcoma 180-bearing mice (in vivo)	Inhibition of tumor growth by inhibiting vascularization in tumor masses	Zhang et al. (2006)

TABLE 14.5 Antimetastatic activity of extracts/fractions/compounds from marine macroalgae.

Compound	Species	Target	Mode of action for antiinvasive or antimetastatic activity	References
Fucoidan	*Fucus vesiculosus* (brown seaweed)	4T1 and MDA-MB-231 cells	Reduction of metastatic lung nodules in 4T1 xenograft female Balb/c mice. Regulation of epithilial-mesenchymal transition via modulation of TGFR/Smad-dependent signaling	Hsu et al. (2014)
Fucoidan	*Fucus distichus* ssp. *evanescens* (as *Fucus evanescens*) (brown seaweed)	Lewis lung adenocarcinoma in mice	Reduced levels of cathepsin L. inhibition of lymph node invasion	Alekseyenko et al. (2007)
Fucoidan	*Undaria pinnatifida* sporophylls (brown seaweed)	Hca-F cell lines and in mouse	Reduced metastasis in vivo. downregulated (VEGF) C/VEGF receptor 3, hepatocyte growth factor/c-MET, cyclin D1, cyclin-dependent kinase 4, phosphorylated (p) phosphoinositide 3-kinase, p-Akt, ERK 1/2, and NF-κB, and suppressed adhesion and invasion by downregulating L-Selectin, and upregulating protein levels of tissue inhibitor of metalloproteinases (TIMPs)	Wang et al. (2014b)
Protamine/ fucoidan nanoparticles	–	MDBA-MB-231 cell line	Enhanced P-selectin mediated endocytosis, charge conversion and stimuli-tunable release	Lu et al. (2017)
Fucoidan	*Fucus vesiculosus* (brown seaweed)	Lewis lung carcinoma cells in mice	Represses lung tumor in mice. Downregulated MMPs, NF-κB and VEGF	Huang et al. (2015)

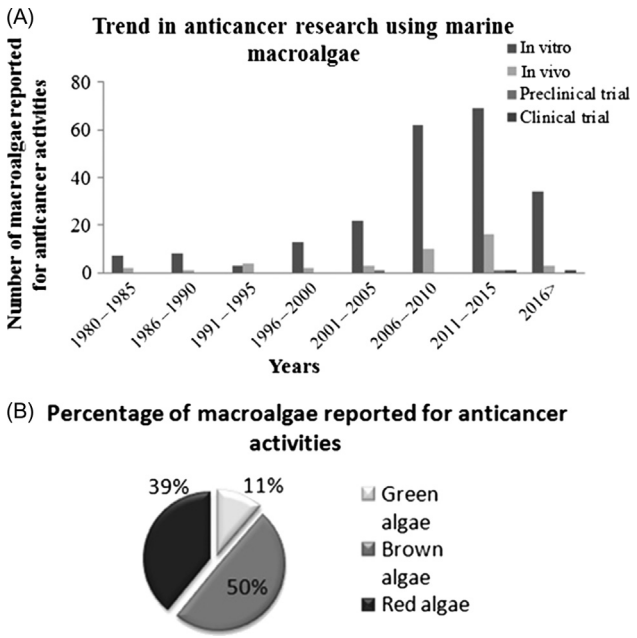

FIGURE 14.1 (A) The number of marine macroalgae that were reported for anticancer activities as from 1980 and onward. Data retrieved from Google Scholar, Science Direct, Plos One, and PubMed, $n = 304$ (number of articles = 211). (B) Percentage of macroalgae reported for anticancer activities. Data retrieved from Google Scholar, Science Direct, Plos One, and PubMed, $n = 304$ (number of articles = 211).

(Table 14.1). The ability of macroalgae to inhibit the transformation (antineoplastic activity) of normal cells into cancer cells has been investigated through transformation assay (soft agar assay) to gain new insights into the mechanism of tumor cell suppression, and thereby advocating its possible use in the prevention and treatment of tumors (Lee et al., 2008). Fucoidan isolated from *Saccharina gurjanovae* (as *Laminaria gurjanovae*) showed transformation inhibition activity

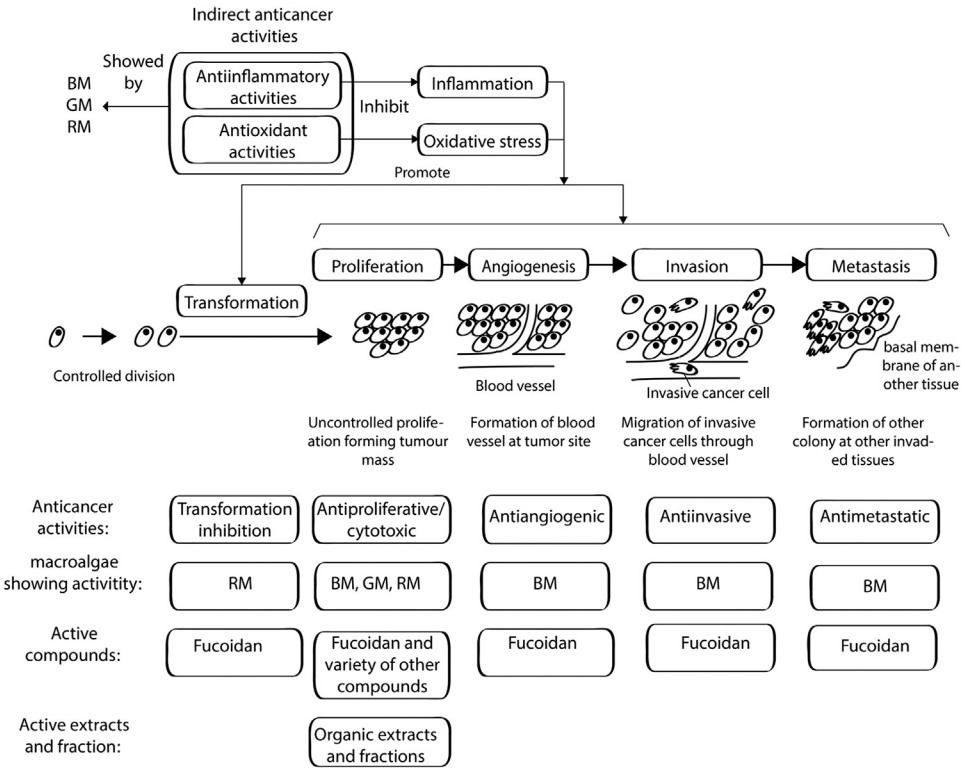

FIGURE 14.2 A summary of direct and indirect anticancer activities of marine macroalgal species. Varied extracts, group of compounds, and compounds of algal species, described from RM—red macroalgae, BM—brown macroalgae, and GM—green macroalgae species, have shown direct anticancer effects, including transformation inhibition, antiproliferation/cytotoxic, antiangiogenic, antiinvasive, and antimetastatic effects by counteracting processes such as transformation, proliferation, angiogenesis, invasion, and metastasis, respectively. Macroalgal species also have the potential to indirectly inhibit cancer through antiinflammatory and antioxidative effects, which indirectly inhibit cancer by reducing oxidative stress and cellular inflammation.

through anchorage independent transformation (soft agar) assay, by inhibiting phosphorylation of epidermal growth factor receptor (EGFR), followed by suppression of extracellular signal-regulated kinase or c-jun N-terminal kinases induced by EGF. Moreover, c-fos and c-jun transcriptional activities were inhibited, resulting in suppression of activator protein-1 (AP-1) activity (Lee et al., 2008).

14.3.1 *In vitro* antitumor activities of marine macroalgae

Cytotoxicity designates the ability of a particular agent to kill cells, while antiproliferative activity delineates the inhibition of tumor growth and cell proliferation by the agent. Most commonly employed assays used to determine cell viability against treatment of macroalgal extracts and compounds have included the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Other dyes often used for cell viability assays are XTT, MTS, WST, and LDH. Polyphenol-rich ethanolic or methanolic extracts, have displayed cytotoxic, antiproliferative or growth inhibitory activities in vitro against a variety of cancer cell lines (Guedes et al., 2013; Hsu et al., 2014; Mhadhebi, Laroche-Clary, Robert, & Bouraoui, 2011; Nwosu et al., 2011; Stanojković et al., 2013; Yeh, Tseng, et al., 2012; Yeh, Yang, et al., 2012). Fractions of *Chondria dasyphylla* (Khanavi et al., 2012) were found to be cytotoxic against different cancer cell lines. Compounds like fucosterol (Khanavi et al., 2012), heterofucans (Costa et al., 2011), fucoidan (Thinh et al., 2013) from brown macroalgae and bromophycoic acids (Teasdale et al., 2012), and halomon (Fuller et al., 1994) from red macroalgae, demonstrated cytotoxic, antiproliferative, or growth inhibitory activities against different human cancer cell lines (Table 14.1).

14.3.2 *In vivo* antiproliferative and cytotoxic activities

Compounds isolated from brown macroalgae, such as fucoidan (Synytsya et al., 2010), alginate (de Sousa et al., 2007) and meroditerpenoids from *Stypopodium flabelliforme* (Pereira et al., 2011) have shown cytotoxic/antiproliferative effects against tumors in vivo (Table 14.1). A 100 mg/kg and 200 mg/kg dose of sulfated polysaccharides inhibited tumor growth by 48.15% and 52.07% respectively, while 50.54% inhibition of the tumor growth was achieved by positive control cyclophosphamide (CTX). In addition to compounds, molecules, such as extracellular polysaccharides (EPS) of the microscopic red alga *Porphyridium cruentum*, showed in vivo antiproliferative activities. At doses of 50, 100, and 200 mg/kg (EPS), administered for 10 days, tumor inhibition index was 20.45%, 49.45%, and 53.33% respectively, while 20 mg/kg CTX showed a 59.38% tumor inhibition rate (Sun, Wang, & Zhou, 2012). Treatment of 200 mg/kg of

extracts of *Sargassum wightii* (Synomym species to *Sargassum schwartzii*) for 14 days against Dalton's ascitic lymphoma (DAL) cells in mice decreased tumor volume to 1.82 ± 0.27 ml compared to the positive control 5-fluorouracil which decreased tumor volume to 1.23 ± 0.58 ml, while in the negative control the tumor volume was 4.89 ± 0.18 mL (Rajan et al., 2013). 100 mg/kg body weight of ethanolic extract of red macroalgae (*Kappaphycus alvarezii*, as *Eucheuma cottonii*) was more effective than tamoxifen in suppressing tumor growth (27%) against LA-7 rat mammary tumor cells developed in rat, improving tissues (plasma, liver, and kidney), malondialdehyde concentrations, superoxide dismutase activity, and erythrocyte glutathione concentrations ($P < .05$) (Shamsabadi et al., 2013). Ethanol extract of *Gracilaria edulis* (300 mg/kg/day) enhanced life expectancy in Swiss albino mice, as 100% survival was found after 28-day treatment, while the control group showed 50% survival at the 32nd day (Patra & Muthuraman, 2013). Orally administered methanol extract of *Gelidiella acerosa* and *Acanthophora spicifera* reduced cancer cell count to $1.88 \pm 0.30 \times 10^6$ and $1.80 \pm 0.25 \times 10^6$, respectively, in comparison to the control $2.70 \pm 0.40 \times 10^6$ (Duraikannu, Shameem Rani, Anithajothi, Umagowsalya, & Ramakritinan, 2014). The administration of 30.4 mg/kg of fucoidan in 0.2 ml per os from *Undaria pinnatifida* (UPF) and *Fucus vesiculosus* (FVF) in a combination of chemotherapy drugs including 10 mg/kg paclitaxel and tamoxifen intravenously in mice inoculated with TOV-112d, MCF-7, or ZR-75 subcutaneously or SKOV3-GFP-Luc ($n = 60$ for four cell lines) for 28 days, led to decreased activity of paclitaxel. The combination of FVF/tamoxifen in the TOV-112d ovarian cancer mouse model had improved activity but there was no difference observed with the UPF/tamoxifen in either ovarian cancer mouse model. No difference was observed with a combination of UPF or FVF with paclitaxel in human ovarian cancer SKOV3 or TOV-112d orthotopic mouse models (Burney et al., 2017).

14.3.3 Induced programmed cell death by macroalgae

Programmed cell death is involved during the induction of cytotoxic activity and antiproliferative activities by marine macroalgae. Apoptosis is the major cell death mechanism employed by several macroalgal extracts (Kim, Yang, et al., 2012; Xue et al., 2012), fractions, and compounds (Tables 14.2 and 14.3). Nevertheless, the usage of other cell death mechanisms, such as anoikis (Go, Hwang, Kim, & Nam, 2011) and autophagy (Hou, Gao, Chen, Hu, & Xie, 2013), have also been described. Different types of extracts, fractions, and compounds from marine macroalgal species have demonstrated the ability to modulate various pathways (Kwon & Nam, 2007; Zhang, Ting-Kong, Wu, & Lan, 2013), receptors, proteins, and genes (Haneji et al., 2005; Kim, Kim, & Nam, 2012; Xue et al., 2012), enhancing DNA fragmentation and sub-G1 arrest (Hosokawa et al., 2004; Namvar et al., 2012), ultimately causing apoptosis (Table 14.3). Most investigations describing apoptosis action mechanism have reported proteins to be modulated upon application of different marine macroalgal extracts and compounds, however, no studies have reported the modulation of oncogenes during the response (Lodish et al., 2000). It is therefore imperative, that further studies determine whether marine autotrophs extracts or compounds have the ability to modulate oncogenes.

The capability of macroalgal compounds to induce autophagy and anoikis, has also been reported. Fucoidan isolated from brown seaweed was reported to enhance cytotoxicity by autophagy (Hou et al., 2013). A glycoprotein isolated from *Saccharina japonica* (as *Laminaria japonica*) was found to induce anoikis in HT-29 cells. It was identified by features like detachment of cells from each other and from the plate and through Western blotting, as expression of matrix metallopeptidase 2 (MMP-2) and MMP-9 increased and expression of integrins and phosphorylation of FAK (focal adhesion kinase) and Src (proto oncogene tyrosine protein kinase) decreased (Go et al., 2011).

14.4 Antiangiogenic activity of marine macroalgae

Angiogenesis refers to the formation of new blood vessels and is regarded as a hallmark of states such as tumor progression (Carmeliet & Collen, 1998; Potente, Gerhardt, & Carmeliet, 2011). Fucoidans have demonstrated antiangiogenic activities both in vitro and in vivo. The antiangiogenic ability of macroalgal extracts/compounds have been determined using chorioallantoic membrane (Cam) assays and adaptation of Cam assays, for, for example, chicken yolk sac membrane assay (Dias et al., 2008) and matrigel assay using calcein-AM vital staining (Dithmer et al., 2014), and growth factors expression (Western blot) (Guerra Dore et al., 2013; Liu et al., 2012). A general ability to inhibit or decrease secretion of a key growth factor involved in angiogenesis, VEGF (Ribatti, 2005; Stringer, 2006), was shown by various marine macroalgal extracts/compounds (Table 14.4). Decrease of microvessel formation and downregulation of hypoxia inducible factor 1 alpha (HIF-1α) substantiated the antiangiogenic effects of extracts/compounds obtained from marine macroalgae (Liu et al., 2009; Salem & Ibrahim, 2011) (Table 14.4). However, it must be noted that the properties of fucoidans vary according to their molecular weight. High-molecular-weight sulfated polysaccharides were found to be antiangiogenic, while, low-molecular-weight fractions were potentially found to show proangiogenic effect. The effects of other fine structural details of fucoidans on angiogenesis are still to be identified (Ustyuzhanina et al., 2014).

14.5 Antiinvasive and antimetastatic activity of marine macroalgae

Tumor metastasis is a multistep process by which cancer cells from the primary tumor enter the vasculature and circulate, migrate, and invade distant secondary organs or tissues (Wang et al., 2014). Marine macroalgal extracts and compounds have been found to inhibit different steps of metastasis (Table 14.5). Cell adhesion and cell invasion assays (Wang et al., 2014), were commonly used to demonstrate antiinvasive ability of compounds and extracts of marine macroalgal species. While other assays and methods, for example, wound healing assay, gelatin zymogen (Hsu et al., 2014), ELISA, Western blotting, and matrigel migration assay (Choi, 2010), have also been employed to demonstrate antiinvasive ability of marine macroalgal compounds. Along with the antiinvasion activity, some studies even demonstrated the antimetastatic activity of macroalgal compounds in vivo (Alekseyenko et al., 2007; Go, Hwang, & Nam, 2009; Hsu et al., 2014; Wang et al., 2014). The inhibition of matrix metalloproteinase 2/9 (MMP-2/9) was demonstrated by fucoidan (Choi, 2010) and glycoprotein during both antiinvasive and antimetastatic activities. Marine macroalgal extracts were able to decrease the expression of proteins such as claudin, and genes, for example, TGFR I and TGFR II (Hsu et al., 2014), and increase the levels of thrombospondin, TIMP 1 and 2, E-cadherin TGF-β (transforming growth factor) and receptors (TGFR) degradation (Kim, Kim, & Nam, 2013), leading to antimetastatic activity.

14.6 Clinical trials

Recently, fucoidan (from *Sargassum hemiphyllum*) has shown positive results in clinical trials. In a double-blind controlled clinical trial, 54 patients given first-line treatment of folicinic acid, 5-flurouracil, irinofecan, bevacizumab therapy, biweekly, were separated into two groups, with the study group treated with 4 g of fucoidan and the control with 4 g of cellulose. Fucoidan treatment was associated with a higher disease control rate, improved overall survival rate, and progression-free survival (Tsai, Tai, Huang, Chang, & Wang, 2017). In another study, patients (10), given oxaliplatin plus 5-fluorouracil/leucovorin or irinotecan plus 5-fluorouracil/leucovorin chemotherapy as first-line treatment, were treated with 4.05 g fucoidan (*Cladosiphon okamuranus*) daily. It was associated with a reduction in the clinical toxicity indicator "fatigue" in comparison to those not taking fucoidan, as well as an increase in tolerance to more rounds of chemotherapy (Ikeguchi et al., 2011). Fucoidan has no significant effect on pharmacokinetics of tamoxifen and letrozole in breast cancer patients, whilst being safe as a complementary medicine (Tocaciu et al., 2016).

14.7 Structure–function relation of anticancer compounds isolated from marine macroalgae

A number of compounds have been isolated from macroalgal species (Table 14.1). Marine polysaccharides have gained much attention in anticancer investigations. Fucoidans are mainly made up of sulfated fucopyranosyls and galactofucans moieties Fig. 14.3 (A) (Ale, Maruyama, Tamauchi, Mikkelsen, & Meyer, 2011). Cytotoxic and antiproliferative activities of fucoidans have been reported and have been linked to existing oversulfation, the positioning of sulfate groups, and their molecular weight (Ale et al., 2011; Cho, Yang, Kim, & You, 2010). Alginate (B) comprises a family of linear polysaccharides which possesses 1,4-linked β-D-mannuronic and α-L-glucuronic acid residues, having the ability to chelate metal ions (Skjåk-Braek, Grasdalen, & Larsen, 1986).

Besides polysaccharides, interesting macrolides have been isolated from macroalgal species. For instance, lobophorolide (C) (Fig. 14.3) isolated from brown seaweed *Lobophora* sp. is uniquely bound to actin, forming a dimerization interface that is composed of the macrolide region. This binding inhibits microfilament stability and holds important implications in the development of actin-targeting drugs and the evolution of macrolide biosynthetic enzymes (Blain et al., 2010). Lophocladines A and B (D) are two 2,7-naphthyridine alkaloids, isolated from the marine red alga *Lophocladia* sp. Lophocladine A showed affinity for N-methyl-D-aspartate receptors and was found to be a δ-opioid receptor antagonist, which holds potential in cancer therapy (Singleton, Moss, Karp, Atkins, & Janku, 2015). On the other hand, lophocladine B showed significant cytotoxicity against human lung tumor and breast cancer cell lines, correlated with microtubule inhibition (Gross et al., 2006). Halomon (E) is a polyhalogenated monoterpene isolated from *Portieria hornemanii*, which shows very interesting cytotoxic properties (Fuller et al., 1994).

14.8 Inhibition of carcinogenic factors

Factors such as oxidative stress (Behrend, Henderson, & Zwacka, 2003), cellular inflammation (Balkwill, 2006; Lu, Ouyang, & Huang, 2006), mutagenic substances (Okai, Higashi-Okai, Nakamura, Yano, & Otani, 1994;

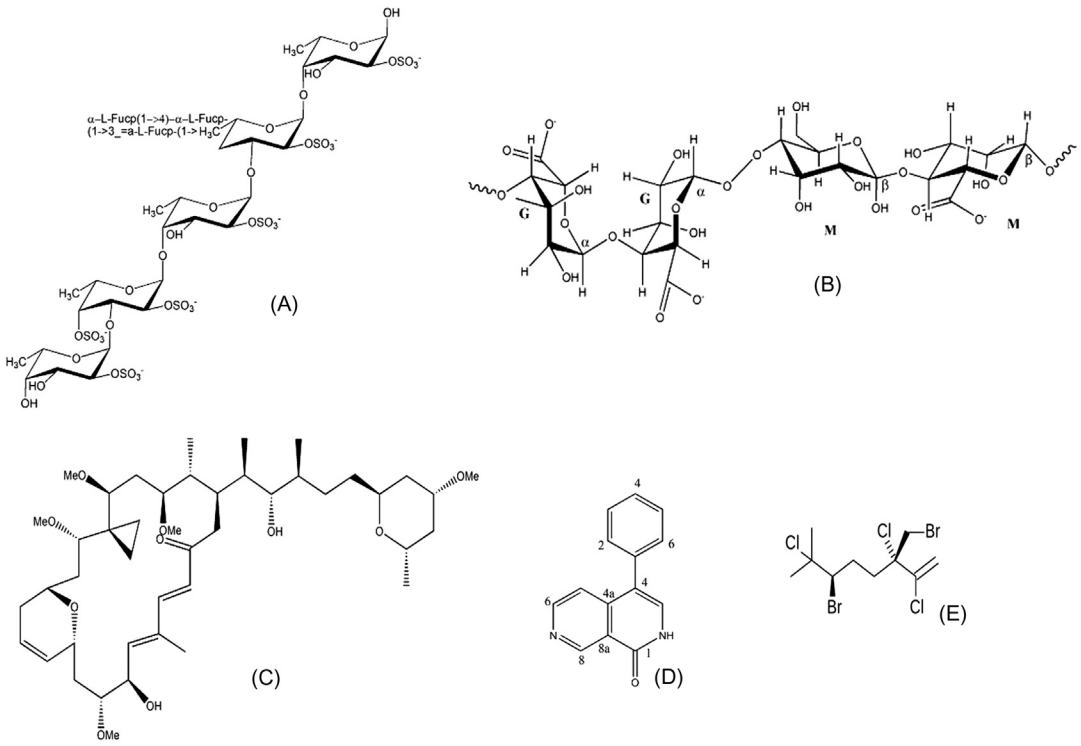

FIGURE 14.3 The structure of compounds isolated from macroalgal species fucoidan (A) (Ale et al., 2011), alginate (B) (Chandía, Matsuhiro, & Vásquez, 2001; Skjåk-Braek et al., 1986), lobophorolides (C) (Blain, Mok, Kubanek, & Allingham, 2010), laphocladine A and B backbone (D) (Gross et al., 2006), and halomon (E) (Fuller et al., 1994) isolated from macroalgal species.

Okai, Higashi-Okai, Yano, & Otani, 1996), and hypoxia (Bohle, Mackensen-Haen, & Wehrmann, 1996; Desmouliere, Guyot, & Gabbiani, 2004) are known to play a key role in initiating cancer and sustaining different cancer processes. In recent years, there has been much effort to find cytotoxic agents that can target oncogenes and tumor suppressor genes. Recent studies have suggested that targeting metabolism, aneuploidy, and immune surveillance instead may also be a promising approach (Gorrini, Harris, & Mak, 2013). Excessive oxidative stress is known to damage DNA proteins and lipids (Behrend et al., 2003; Gorrini et al., 2013). Antioxidants play an important role in controlling radicals such as ROS, and thus controlling oxidative stress (Boonstra & Post, 2004; Cadet, Douki, & Ravanat, 2010; Conklin, 2000). Antioxidants were thus hypothesized to be used in the management of ROS so as to control cancer. ROS have also been reported to be involved in the regulation of angiogenesis by regulating the expression of VEGF (Ye, Wang, Zhou, Liu, & Zeng, 2008). However, the role of ROS in cancer management is complex as they can be both tumor suppressing and oncogenic depending on concentration (Trachootham, Alexandre, & Huang, 2009).

Antioxidants have been described from various marine organisms around the world, such as macroalgae (Ramah et al., 2014; Zubia, Robledo, & Freile-Pelegrin, 2007). Various nonmacroalgal sources of antioxidants have been proven to retain antitumor properties (Hurst et al., 2012; Jaramillo & Zhang, 2013; Richman & Chan, 2012). Nuclear factor E2-related factor 2 (Nrf2)−antioxidant-response element (ARE) signaling pathway is often used in response to oxidative stress (Zucker et al., 2014). An acidic compound isolated from the green seaweed *Ulva lactuca* induced the expression of ARE-regulated cytoprotective genes, as well as Nrf2 activation in IMR-32 human neuroblastoma cells and in mice (Wang, Paul, & Luesch, 2013). Marine antioxidant-rich organisms may thus be sources of potent anticancer activities.

An inflammatory microenvironment is involved in the development of cancers (Balkwill, 2006; Coussens, 2002; Philip, Rowley, & Schreiber, 2004). At molecular level, extracts of seaweeds have been reported to downregulate the expression of the inflammatory cytokine TNF-α, to enhance anticancer effect (Hwang, Chen, Yang, Wang, & Chan, 2011; Jayasooriya, Moon, Choi, Yoon, & Kim, 2011). Such extracts warrant further studies, so as to determine the class of compounds or individual compounds leading to downregulation of the expression of TNF-α and their detailed mechanism of actions in malignant cells. Hypoxia is involved in cell proliferation and tumor onset and development (Bohle et al., 1996; Desmouliere et al., 2004). Compounds such as caulerpin from green seaweeds and fucoidan from brown

seaweed have shown the ability to induce activation of HIF-1, which is known to be a master regulator of cellular adaptation to hypoxia (Blouin, Pagé, Soucy, & Richard, 2003; Higgins et al., 2007; Teng et al., 2015), blocking HIF-α protein (Liu et al., 2009) and controlling nuclear levels of NF-κB (Lee, Kim, & Kim, 2012), respectively, to inhibit hypoxia-induced damages.

Moreover, macroalgae have the ability to inhibit cancer by inhibiting antimutagenic agents. For instance, Pheophytin a isolated from *Ulva prolifera* (as *Enteromorpha prolifera*) and *Pyropia tenera* (as *Porphyra tenera*) showed antimutagenic activity on mutagen-induced umu C gene expression in *Salmonella typhimurium* (TA 1535/pSK 1002) (Higashi-Okai, Otani, Okai, & Hiqashi-Okaj, 1999; Okai et al., 1994, 1996). β-Carotene isolated from *P. tenera* exhibited antimutagenic activity (Okai et al., 1996). *Hypnea musciformis* extract possesses strong antigenotoxic, anticlastogenic, and protective effects against genotoxic/mutagenic agent mitomycin C in vitro. Marine organisms with antioxidant activities, antiinflammatory activity, hypoxia-inhibiting ability, and antimutagenic activity may thus represent potential sources for anticancer activities. Such compounds from marine macroalgae must thus be considered in further exploration of anticancer activities in macroalgae.

14.9 Impact of physical and environmental factors on anticancer activities

In marine ecosystems factors such as light, tidal regime, nutrients, temperature, salinity, water movement, predation, and desiccation stresses affect marine organisms (Helmuth, Mieszkowska, Moore, & Hawkins, 2006; Hu, Li, Sommerfeld, Chen, & Hu, 2008; Parida & Das, 2005; Shi, Sui, Wang, Luo, & Ji, 2005). Moreover, different environmental conditions and ecological niches existing during different seasons lead to differential adaptations of these organisms (Barnes & Hughes, 2009; Nybakken, James, & Bertness, 2005). As an example, the yield and composition of fucoidan increases in summer. Skriptsova, Shevchenko, Zvyagintseva, and Imbs (2010) showed that crude fucoidan content of the brown seaweed *U. pinnatifida* can vary from 3.2% to 16% of dry weight. In addition, the chemical composition, including levels of sulfation, in fucoidan varies seasonally (Imbs et al., 2009; Skriptsova et al., 2010). Production of fucoidan was accentuated during slight blade decaying and sporangia maturation, which follows a seasonal pattern (Honya, Mori, Anzai, Araki, & Nisizawa, 1999). In marine organisms, ROS levels are affected by the physical conditions of the organisms and environmental conditions (Legrand, Rengefors, Fistarol, & Granéli, 2003). Marine organisms that experience high levels of oxidative stress enhance the production of secondary metabolites. This helps marine organisms to survive oxidative stress by scavenging harmful radicals (Connan, Deslandes, & Gall, 2007; Cragg, Kingston, & Newman, 2005; Pereira et al., 2011). It is notable that variations of secondary metabolites or compounds showing anticancer activities have been related to environmental conditions and seasonality. Further studies in this field may contribute to the identification of more efficient and diverse metabolites with possible anticancer properties.

Both inter- and intraspecific variations in yield, bioactivity, composition, and structural traits of compounds isolated from marine macroalgae are known (Ale et al., 2011). For instance, fucoidan varies in different species, in terms of sulfate contents and substitutes and the presence of sugar moieties, for example, galactose, mannose, xylose, and glucose in addition to fucose (Jiao, Yu, Zhang, & Ewart, 2011; Rani, Shakila, Jawahar, & Srinivasan, 2017). The composition of fucoidan isolated from *Fucus serratus* differed from *Fucus distichus* (Skriptsova et al., 2010). Yields of fucoidan vary among different brown macrolalgae species, for example, *Padina tetrastromatica* yielded the highest amount of fucoidan, followed by *Turbinaria ornata* and *S. wightii* (Rani et al., 2017). Proper identification of macroalgae is thus crucial at the commercial level. The identification of macroalgae can be very difficult, and indeed the whole taxonomic system of these organisms is undergoing a major scientific revolution (Bolton, 2019, De Clerck, Guiry, Leliaert, Samyn, & Verbruggen, 2013). Many of the macroalgae species names used in the literature have recently changed, as shown by many examples in this paper, where names have been updated. Most of the changes are ascribed to use of molecular sequencing in taxonomy and identification over recent decades. In addition, there are many regions of the world where the macroalgal flora is poorly studied, and important investigations may be carried out on chemical and medical properties using biological material, which are not repeatable due to misidentification and poor documentation. It is imperative not only that specimens are preserved in a recognized facility, but also it is increasingly clear that DNA markers (barcodes) should be obtained of specimens used, wherever feasible.

14.10 Prospective for anticancer research in seaweeds

There are many prospective avenues for anticancer studies of marine macroalgae (Fig. 14.4). With less than 1.5% of worlds macroalgae species assessed, it is imperative that maximum macroalgal species are screened for their anticancer

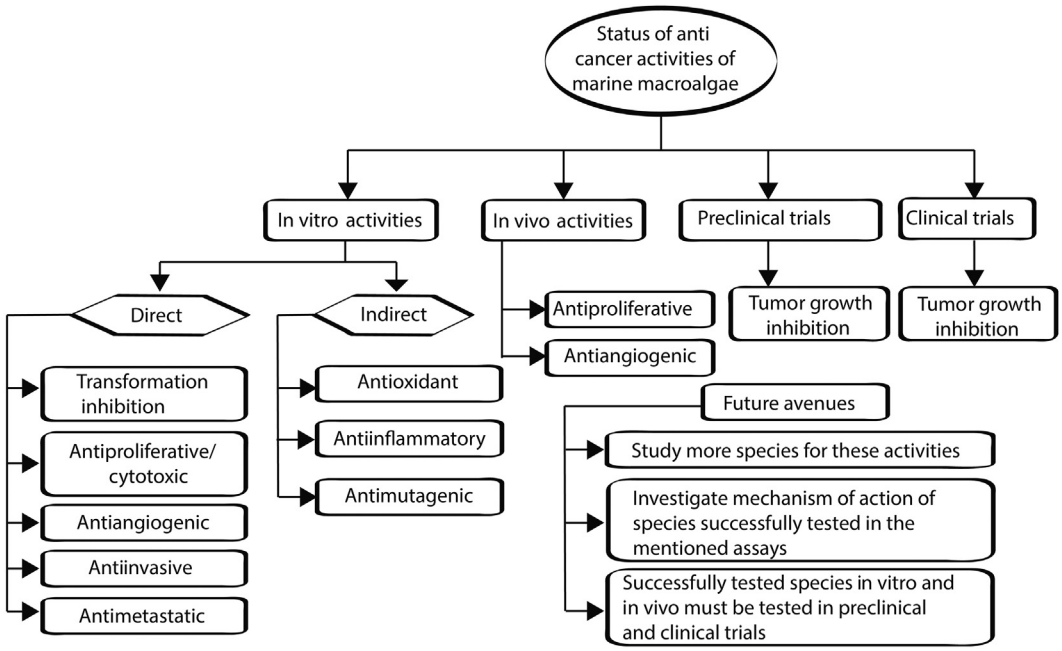

FIGURE 14.4 Prospective avenues for anticancer studies of marine macroalgae. Actual status of various anticancer activities (direct and indirect) exhibited by macroalgal molecules are summarized. The future directions in respective fields of study are also needed.

potential. These potential extracts or fractions need to be further characterized to provide more accurate information on the active compounds present. Active compounds or groups of compounds producing anticancer effects must be isolated and identified. Furthermore, after isolation, outlining of synergistic, potentiating, or antagonizing effects of the groups of compounds is vital. Potential compounds may also be tested for combined effects with other existing anticancer drugs, as macroalgal compounds have been shown to enhance the ability of synthetic drugs (Zhang, Teruya, Yoshida, Eto, & Shirahata, 2013). Extracts tested successfully through *in vitro and in vivo* assays must have their mechanisms of action demonstrated and further tested through preclinical and clinical trials, by overcoming challenges such as financial, supply, and technical limitations (Martins et al., 2014). It is also important to determine the ability of marine organisms to modulate oncogenes, which will provide more insight into the anticancer potential of extract or compound. For instance, it has been proposed that the ability of fucoidan to downregulate the EGFR/KRAS/BRAF pathway is further investigated to confirm its possible anticancer mechanism against colon cancer (Tsai et al., 2017). Furthermore, marine organisms with antioxidant, antiinflammatory, hypoxia-inhibiting, and antimutagenic activities may represent potential sources in cancer chemoprevention or management, and must be given due consideration in marine anticancer research programs. In addition, the determination of environmental effects on the yield of secondary metabolites in marine organisms exhibiting anticancer activities is critical.

14.11 Conclusion

Following efforts to find anticancer cures or preventive measures against cancer, marine macroalgae have emerged as highly hopeful sources of anticancer activities. Followed by the success of fucoidan isolated from brown macroalgae in eliminating cancer cells, halting proliferation, angiogenesis, cell invasion, and metastasis, various extracts, fractions, and compounds have been explored for their ability to inhibit transformation, for their cytotoxic, antiproliferative, and growth inhibitory effects against cancer cell lines, *in vitro* and *in vivo*. Studies of the molecular mechanisms attributed to some of these activities have also been delineated, thus assisting in identifying molecular targets, thereby justifying the relevance of their effect. The potential of marine macroalgae in cancer research is high and further investigations of the studied species, as well as preliminary anticancer investigations using unexplored marine organisms are to be encouraged. Interesting compounds must ascertain their efficacies through preclinical and clinical trials. Indirect anticancer works on the implication of oxidative stress, inflammation, mutation, and hypoxia would warrant further exploration in the quest of managing cancer. In addition, genetic variations studies, in terms of inter- and intraspecific

variations in yield and compound structures exist, though very scarce, thus necessitating further works. The variation of anticancer activities with respect to physical and environmental conditions is a domain still to be delved into.

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Chapter 15

Insights into the bioactive compounds of endophytic fungi in mangroves

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15.1 Introduction

The fungi are known to possess exclusive capabilities allowing them to colonize different habitats, ranging from terrestrial to freshwater and marine (Redou, Navarri, Meslet-Cladière, Barbier, & Burgaud, 2015). Their number is estimated to lie at around 1.5 million species based on a fungus to plant ratio of 6:1 (Frohlich & Hyde, 1999). However, modern high-throughput sequencing methods put this number at around 5.1 million species (Blackwell, 2011). Out of these, the marine species constitute roughly 1500, as recorded by Hyde and Soytong in 1998. This number does not include the lichens and other fungi that are not yet fully described. Out of this high number, only 120 species have been listed by Hyde globally from 29 mangrove forests (Deshmukh & Balaji, 1994), but their number is increasing annually (Kerry et al., 2018). Different demographic sites and regions provide researchers with opportunities to record unique new species with diverging distributions limited to unambiguous niches and ecosystems (de Souza et al., 2013). These species might offer several benefits due to the bioactive compounds they potentially produce, particularly the species originating from extreme environments such as mangrove sites.

According to Ross and Adam (2013), de Souza et al. (2013), and Hanum, Latiff, Hakeem, and Ozturk (2014) the mangroves are wetland ecosystems. These are located between the land and sea, comprising plants, animals, fungi, bacteria, microalgae, and invertebrates inhabiting coastal areas and river estuaries. They are ranked second among the most productive and precious marine ecosystems (Hanum et al., 2014; Salem & Mercer, 2012), undertaking a precarious role as a natural buffer zone between sea and land, because of their strong roots and buttress systems. They also tend to break high winds and wave actions as has been proved during tsunamis particularly the one which struck the areas of Kedah, Perak, and Langkawi (Malaysia) in 2004. The mangrove trees are at the same time harvested for making poles and used for charcoal production as well. All such uses benefit the locals in various aspects, especially their economy. The mangroves also regulate the sediment movement. The streams and rivers flowing into the mangrove areas lose their flow speed giving space and time for the sediments to settle down at the bottom and ultimately allowing the movement of sediment-free water into the sea. The water-borne pollutants many times become hazardous for living beings but mangroves around the area can act as sinks and prove beneficial for health. The mangrove soils and roots are reported to successfully trap and immobilize heavy metals and nutrients from wastewater reaching there from the hinterland (Latiff & Faridah-Hanum, 2014). This function results from the close interaction between plants, microbes, and nutrients within the mechanism of recycling and conserving nutrients in such ecosystems (Shakilabanu, Kanchana, & Jayanthi, 2012). The major contributor for the nutrient transformations in mangroves is the microbial community with 91% of the total microbial biomass comprising bacteria and fungi (Ghizelini, Mendonça-Hagler, & Macrae, 2012). Recently, an inclusive culture-based method has led to the isolation of 183 deep subsurface marine fungal species (Redou et al., 2015). The antimicrobial screening of 110 species among them has emphasized that 33% of the tested strains show antimicrobial activity (Navarri et al., 2016).

Several fungal species are associated with different ecosystems as epiphytes, pathogens, and endophytes. The fungal endophytes are those residing inside the host tissues without causing any damage to the host

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(Aly, Debbab, Kjer, & Proksch, 2011; Botella & Diez, 2011). The relationship of such fungi varies in response to the genotypic characteristics and environmental conditions of the host (Singh, Gill, & Tuteja, 2011). These endophytes commonly show a symbiotic relationship with their host, a state which favors metabolic interactions with them and their environment leading to the synthesis of a great variety of bioactive secondary metabolites. The advantages of such partnerships are that these secondary metabolites enable the host to resist the external stresses, both biotic as well as abiotic (Dutta, Puzari, Gogoi, & Dutta, 2014; U'Ren, Lutzoni, Miadlikowska, Laetsch, & Arnold, 2012). A study conducted by Meena et al. (2017), suggests that under extreme environmental conditions, plants show more interesting metabolite production related to their fungal endophytes. The endophytic fungi have nearly the same metabolic pathway as their host, which allows the fungi to produce metabolites that mimic the structure and function of phytochemicals of the host (Kusari, Hertweck, & Spiteller, 2012). A combination of morphotyping and ITS (internal transcribed spacer) sequencing resulted in the recognition of endophytic fungi in *Dendrobium officinale* (Jin et al., 2017). Currently, much research is being carried out on metabolite extraction from fungal endophytes with the aim of discovering structurally novel and potent bioactive molecules (Liu et al., 2016).

Plants growing in association with endophytes often grow faster than plants without endophytes. This rapid growth may be due to the production of phytohormones like indole-3-acetic acid (IAA), cytokines, and other plant growth−promoting substances (Abdou, 2011; Etesami, Alikhani, & Hosseini, 2015). Some endophytes produce phytochemicals which improve the environmental adaptability of the host. The reason being that they enhance their tolerance to environmental stresses, resistance to phytopathogens, and herbivores including insects (Abdou, 2011; Owen & Hundley, 2004). Endophyte fungi−bound plants usually possess a higher tolerance to drought and aluminum toxicity when compared to plants without endophytic fungi (Clay & Schardl, 2002). Some endophytes are able to boost up the host allelopathic effects on adjacent species to minimize competition for nutrients and space (de Souza, Ambrosini, & Passaglia, 2015). According to Abdou (2011) and Nisa et al. (2015) this could be one of the reasons that some plants with special endophytes are usually competitive enough to dominate a particular area. This review covers the useful information about endophytic fungal mangroves with novel bioactive compounds and the biological processes related to them up to the recent times.

15.2 Endophytic fungi colonization in marine environment

The incredibly diverse ubiquitous asymptomatic microbes like fungal endophytes may be symbiotic or nonsymbiotic. Yokoya, Postel, Fang, and Sarasan (2017) state that the endophyte diversity may confer fitness benefits to plant communities depending on abiotic conditions and the genotype of plants. According to Banerjee (2011), more than 80 genera of endophytic fungi have been isolated from tropical and subtropical plants. Many studies have shown that a significant number of plants have acquired endophytic fungi for their adaptation to survive in their natural environment which imposes a significant stress on the growing conditions for many organisms (Debbab, Aly, & Proksch, 2011, 2012; Tejesvi, Kajula, Mattila, & Pirttilä, 2011). Endophytes colonize not only land-dwelling plants but also marine algae (McMullan-Fisher et al., 2011), mosses (U'Ren et al., 2012), and ferns (Del Olmo-Ruiz & Arnold, 2014). Their distribution in tropical and temperate regions is reported to be very broad (Arnold, Maynard, & Gilbert, 2001; Hyde & Soytong, 2008; Zhang, Rossow, & Stackhouse, 2006; Zimmerman & Vitousek, 2012). Seventy percent of the planet's surface is covered by marine ecosystems and these are still an underexploited source of valuable metabolites. Among microbes, filamentous fungi are fascinating organisms used for the production of various secondary metabolites with a wide application in industries (Fouillaud et al., 2017).

Venkatachalam, Mb, Thirunavukkarasu, and Suryanarayana (2015) have succeeded in extracting 117 endophytic isolates, of which 73 belong to seaweeds and 44 to seagrasses from Southern India. The isolated fungi include the species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Nigrospora*, *Pestalotiopsis*, *Penicillium*, and several more genera of which some are a potential source of chitin-modifying enzymes. Similarly, de FelIcio et al. (2015) have conducted a study in Brazil and have succeeded in isolating species like *Trichoderma atroviride*, *Nigrosporaoryzae*, *Phomopsis* sp., *Penicillium decaturense*, *Xylaria* sp., and *Acremonium implicatum* isolated from Brazillian marine red alga *Bostrychia tenel*. Earlier de Oliveira, de Felício, and Debonsi (2012) conducted a survey and reported that nearly 80 unknown bioactive compounds isolated from endophytic fungi are associated with marine macroalgae. This stresses the fact that an incredible chemical and biological diversity originates from this less explored source. Up until 2009, 2840 marine species had been studied, leading to the extraction of 20,057 compounds (de FelIcio et al., 2015). However, despite the high number of the recognized marine species, only 1% of them has been investigated in this connection (Blunt, Copp, Keyzers, Munro, & Prinsep, 2013).

15.3 Mangrove endophytic fungi

Mangrove-specific growth conditions make fungi adapt themselves to highly stressful environmental conditions leading to the activation of some interesting metabolic pathways, synthesizing distinct, potent biomolecules that allow them to endure the extreme conditions (Corinaldesi, Barone, Marcellini, Dell'Anno, & Danovaro, 2017; de Souza et al., 2013). Mangrove–fungi interaction in an unambiguous environment has led to the discovery of many bioactive compounds (Blunt et al., 2013). There was a significant impact of fungal endophytes on drug discovery, reducing the dependency and burden on plants alone (Deshmukh, Prakash, & Ranjan, 2017). The production of metabolites is incumbent for supporting the survival of the fungal species growing under extreme conditions of the marine environment (Marx, Carpenter, & Deming, 2009). There is a lack of information on secondary metabolites owned by fungi from the marine environment, especially mangroves (Bajpai, 2016). An interesting study has been carried out by Zainuddin et al. (2010) to investigate the antimicrobial characteristics of 152 marine-derived fungi from Malaysia. They have isolated a novel compound named 2,2,7-trimethyl-2H-chromen-5-ol from the marine-derived fungus, *Fasciatis poranypae*.

15.4 Distribution of mangrove endophytic fungi

de Souza et al. (2013) has isolated 343 endophytic fungi from three mangroves in Brazil, namely, *Rhizophora mangle*, *Avicennia schaueriana*, and *Laguncularia racemosa*. The molecular identification of these fungi has illustrated that they have originated from at least 34 different genera (de Souza et al., 2013). Marine biotopes have been a significant source for fungi belonging to genera such as *Penicillium*, *Paecilomyces*, *Eurotium*, *Fusarium*, *Monodictys*, *Halorosellinia*, and *Alternaria* (Fouillaud et al., 2017).

Diaporthe and *Phomopsis* are dominant fungal genera in the majority of host species that have been investigated (Suryanarayanan, Murali, & Venkatesan, 2002). Apart from these two genera, currently the major genera found in mangroves are *Trichoderma*, *Fusarium*, *Colletotrichum*, and *Xylaria* (de Souza et al., 2013). These genera are widely distributed amongst other mangrove species (Cheng, Chang, & Lee, 2009).

15.5 Factors influencing the endophytic fungal distribution

The composition and dominance of the endophyte species is dependent on the following factors;

1. The host plant species (Botella & Diez, 2011; Higgins, Arnold, Miadlikowska, Sarvate, & Lutzoni, 2007; Stuart, Romao, Pizzirani-Kleiner, Azevedo, & Araujo, 2010).
2. Geographical diversity (Saikkonen, Wäli, Helander, & Faeth, 2004; Thomas, Crozier, Aime, Evans, & Holmes, 2008).
3. Seasonal variations (Arnold & Herre, 2003).

15.6 Environmental condition

Endophytic fungi have been isolated from various mangrove areas with environmental conditions such as:

1. Cananeia, which is a healthier forest with high sediment accumulation rate (SAR); and
2. Bertioga mangrove with an oil spill-affected area and an oil spill-unaffected area.

The endophytic fungal community colonizing each plant species may vary depending on the uniqueness of the fungus–host interaction (de Souza et al., 2013). Root colonization ability also plays a significant role in influencing the plant growth–promoting and biocontrol activity, thereby acting as the first line of defense against the seed- and root-borne phytopathogens (Potshangbam, Devi, Sahoo, & Strobel, 2017).

A healthy mangrove environment might harbor the greatest population of endophytic fungi, but a loss of fungal diversity has been reported in mangroves affected by oil pollution (Hyde, 2004). These results contradict the findings of de Souza et al. (2013). No significant differences in the diversity and richness of fungal communities between oil-affected and normal sites were observed in the experimental data published by de Souza et al. (2013), suggesting that 20 years' time is enough for the mangrove fungal community to recover from an oil spill. A comparison of *A. schaueriana*, *R. mangle*, and *L. racemosa* has revealed that no substantial difference is seen in the density of endophytic fungi between healthy mangrove (Cananeia, Malaysia) and an oil spill-unaffected site (Bertioga, Brazil) (de Souza et al., 2013). These results may be interpreted as that, in both these environments, anthropogenic effects are reduced. Therefore the protection of endophytic fungi acts as an important factor for preserving the mangrove forests.

15.6.1 Season

The diversity and richness of the endophytic fungal communities isolated in summer are greater than in winter (de Souza et al., 2013; Suryanarayanan, Kumaresan, & Johnson, 1998; Suryanarayanan et al., 2002), this suggests that the high humidity and temperature during the summer months (de Souza et al., 2013) could be favorable for the fungi to grow and colonize the plants, which eventually leads to an abundance of fungi isolated from mangrove species. However, the highest mangrove endophytic fungi colonization has been reported during the dry season compared to the rainy season (Costa, Zucchi, & De Melo, 2013). The correlation between the species diversity of the endophytic fungi and season has been negated by Frohlich and Hyde (1999).

15.6.2 Host plant factors

Age and biochemical activity of the host plant influence the abundance and distribution of the endophytic fungi (Arnold & Herre, 2003). With an increase in the age of the host, there is an intensification in species richness and abundance of endophytic fungi. The importance of the age factor is ascribed to:

1. the fungi are spread through air, thus older trees are much more susceptible to repeated infection by airborne fungi; and
2. with the aging of trees, the plant physiological status changes, including the fissures on tree bark, exposing the plant tissue and the primary route for fungal invasion (Fisher, Petrini, Petrini, & Sutton, 1994).

The abovementioned things imply that age factor plays a significant role in colonization of endophytic fungi. The differences in the species diversity of endophytic fungi are also host-dependent due to distinct host characteristics (Costa et al., 2013). The species of Rhizophoraceae are rich in tannins, a phenolic substance that may inhibit fungal growth. However, tannins are believed to be nontoxic to fungal species in terrestrial plants. There is an increase in tannin concentration with the aging of leaves and simultaneously the endophytic fungus population increases (Barbehenn & Peter, 2011). A higher colonization of *Trichoderma* sp. has been recorded in mangrove leaves of *Aegiceras corniculatum* compared to other mangroves (Saravanakumar & Kathiresan, 2014). This difference is reported to be mainly due to the biochemical variations among various mangrove species. Hence the biochemical characteristics of the host plant significantly influence the degree of colonization by endophytic fungi (Coley & Barone, 1996).

15.6.3 Variation among different plant parts

The amount of fungal colonization differs in different plant parts. A higher fungal colonization has been recorded in the branches and stems as compared to the leaves (de Souza et al., 2013; Li et al., 2012; Sun, Guo, & Hyde, 2011; Zheng et al., 2013, 2015). Similarly, the bottom parts of the plant have revealed a greater diversity of endophytes, implying that colonization of most microorganisms in the host takes place through roots (Gazis & Chaverri, 2010). However, in a study conducted by Tao, Liu, Hyde, Lui, and Yu (2008), a different finding was reported, that is, that the diversity of endophytic fungi within leaves is higher than roots. In the same way, Kharwar, Mishra, Gond, Stierle, and Stierle (2011) have indicated that endophytic fungi colonization is greater in leaves (72.22%) than stem (67.78%). These results stress the fact that fungal colonization varies according to the plant part. The reason for this variation seems to be related to the nutrient availability in the regions infected by the endophytic fungi (Shrivastava & Kumar, 2015). Another reason could be the nature of the endophytic fungi, because the parts attacked by the enemies such as insects, leads toward their greater accumulation in the stricken areas to protect themselves as well as their host from enemies (Partida-Martinez & Heil, 2011). In general, a great diversity in results regarding endophytic fungal colonization in various host parts is reported.

15.6.4 Crown height and canopy cover

This factor influences the amount of light passing through to the forest floor. A dense cover of mangroves may be responsible for the differences in the colonization. However, currently, mangroves are widely exploited for their products like timber and charcoal. This exploitation results in a change of their canopy cover. Certainly, endophytic fungal communities are affected by various environmental factors (Zimmerman & Vitousek, 2012). Crown height and canopy cover factor appear to be one of the causes affecting the endophytic fungal distribution in a particular area

(Arnold & Herre, 2003). Samples taken from the dense site recorded higher species diversity and abundance of endophytic fungi compared to samples collected from less dense sites (Gazis & Chaverri, 2010; Petrini, 1991).

15.7 Mangrove endophytic fungi are a great source of novel bioactive compounds

Endophytic fungi have been accredited as the most promising source of bioactive compounds for drug discovery, and substantial progress has been made in exploring their diversity, species richness, and bioprospects (Das, Samantray, & Thatoi, 2018; Kaul, Gupta, Sharma, & Dhar, 2017; Kusari, Singh, & Jayabaskaran, 2014). Fungal endophytes from exclusive environmental surroundings offer a pool of potentially useful pharmaceutical entities (Sarasan et al., 2017). The data published during the last two decades stresses the fact that a high diversity of microorganisms producing novel bioactive compounds reside in the plant tissues (Pimentel, Molina, Dionísio, Maróstica Junior, & Pastore, 2011). The discovery of the anticancer drug taxol from the bark of *Taxus brevifolia* has been a great factor pushing in this direction, which has led to an increase in the research interests on endophytes as well (Stierle, Strobel, & Stierle, 1993). Endophytic fungi can produce this same billion dollar compound. Interestingly from the sequencing of the taxadiene synthase gene, it was found that the endophytic fungi follow their self-biosynthetic pathways in producing taxol (Staniek, Woerdenbag, & Kayser, 2008). The endophytes try to activate various metabolic pathways and in turn produce compounds which help them to survive in the host tissues (Vasundhara, Kumar, & Reddy, 2016). During the last few years, the research in this direction has received considerable attention from natural product chemists, particularly for the production of various novel bioactive compounds from marine natural sources (Aly et al., 2011; Pimentel et al., 2011). During 1981−2005, natural products were a reliable source of drug leads with more than 40% of new chemical entities (NCEs) reported as having been derived from microorganisms. Moreover, more than 60% of the anticancer and 70% of the antimicrobial drugs currently used are derived from natural products or natural product derivatives (Newman & Cragg, 2012). A recent study reports that endophytic fungi derived from mangrove plants form an excellent source of potential new bioactive secondary metabolites, and some of them have potent biological activity (Bajpai, 2016; Chagas, Caraballo-Rodriguez, & Pupo, 2015; Das et al., 2018; Kaul et al., 2017). Recently two new phenyl derivatives, two new natural compounds, and three known compounds have been isolated from the *Kandelia candel* fruit, a mangrove species (Ju et al., 2016).

15.8 In-depth study of mangrove endophytic fungi bioactivities

The chemical diversity of the secondary metabolites of mangrove endophytic fungi has proven useful for novel drug discovery. They possess a potential for use as a source of pharmaceutical products, because they possess antiviral, antioxidant, anticancerous, antidiabetic, acetylcholinesterase, and other biological activities. Endophytes live in a special and sequestered environment and represent a less explored ecological group, but are a tremendous source for bioprospecting new drugs (Kaul et al., 2017).

15.8.1 Antimicrobial, antifungal, antiviral, and antimalarial activities

The emergence of antibiotic resistance in various pathogens has become a major concern for every individual as the antibiotics are waning in effectiveness for combating these superbugs. The report published by World Health Organization stated that every year 480,000 people are affected by multidrug resistance (MDR) tuberculosis. Among these, MDR bacteria are identified as the causal agents passing infections from hospitals. It is reported that it is a significant public health threat at present (Revelas, 2012). To find a solution to such problems, researchers are trying many alternatives, and one of these is the use of novel antibacterial compounds from mangrove endophytic fungi.

Fungal endophytes are a rich source of natural products with unique structures with a promising way to overcome the increasing threat of drug-resistant microbes, and can be useful as food preservatives to control food spoilage and food-borne diseases (Das et al., 2018; Kaul et al., 2017; Liu et al., 2007).

Some researchers have isolated several fungal endophytes, including *Colletotrichum* sp., *Alternaria* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Guignardia* sp., and *Cladosporium* sp., from the mangrove *A. corniculatum* (Bin et al., 2014). The fungal species isolated have been tested against some of the human pathogenic bacteria, such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumanii*. The antibacterial activity of *Colletotrichum* sp. against all the five bacteria including the MDR pathogens (*K. pneumoniae* and *A. baumanii*) has been fully confirmed.

Christophersen et al. (1998) have investigated 227 marine isolated endophytic fungi for different levels of antimicrobial activities against *Staphylococcus aureus*, *Vibrio parahaemolyticus*, and *Salmonella typhi*. Endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum* have been recorded to show antimicrobial potential against *Bacillus subtilis*, *Enterococcus* sp., *K. pneumoniae*, *P. aerugionsa*, *S. typhi*, and *S. aureus* (Maria, Sridhar, & Raviraja, 2005). A large number of metabolites isolated from *Talaromyces* endophytic fungus occurring on the stem bark of *K. candel* have antimicrobial activity (Liu et al., 2010).

Recently structural elucidation and biological activities of new metabolites, phomopsin A, B, and C, together with two known compounds, cytosporone B and C, isolated from *Phomopsis* endophytic fungus from the stem of *Excoecaria agallocha* have shown antifungal activity against several fungi (Huang et al., 2008). *Guignardia* and *Neusartoya* endophytic fungal species isolated from the *Avicennia* mangrove taxon have shown strong antifungal activity. These endophytes contain several metabolites (Ling, Teen, Mujahid, Proksch, & Muller, 2016).

Sixty-nine endophytic fungi isolated from six mangrove species, that is, *Avicennia alba*, *Avicennia marina*, *Bruguiera* sp., *Ceriops* sp., and *Sonneratia* sp., have been tested against *Fusarium oxysporum*, a pathogenic fungus causing fusarium wilt in tomato roots (Rahmansyah, 2013). Among the 69 isolates, 22 isolates have shown antagonistic activity against *F. oxysporum*, and *Trichoderma hrzianum* has proven the best among all species. Among these 22 isolates that showed antagonistic nature, some are volatile antifungals and some are nonvolatile agents. After evaluation of antifungal activity, the highest pathogen inhibition among the 13 volatile antifungal agents was demonstrated by *Colletotrichum*. The decrease in growth of pathogen indicates that the endophytic fungus *Colletotrichum* releases the volatile metabolites. Apart from *Colletotrichum* sp., other volatile metabolite producers are *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Talaromyces* sp., and also *Trichoderma* sp. (Suciatmih, Supriyati, & Rahmansyah, 2013). Ting, Mah, and Tee (2010) have reported that *Penicillium* also produced volatile metabolites of butanol, 3-methyl, β-butyrolactone, and 2-butenedinitrile. *Trichoderma* sp. is widely known for its antifungal activity, this could be related to a characteristic of *Trichoderma* sp. which produces many antibiotics and has the ability to produce extracellular enzymes responsible for making it a good biocontrol agent (Rahmansyah, 2013). According to Howell (2003), *Trichoderma* sp. competes for space and gets its food through rhizosphere competence and also boosts the host plant resistance. From the nonvolatile antifungal agent, *Aspergillus niger* recorded the highest zone of inhibition toward pathogenic fungi (Rahmansyah, 2013). The inhibition zone indicates the antibiotics secreted by the endophytic fungi. *A. niger* has also been reported to inhibit the growth of *Rhizoctonia solani* and *F. oxysporum* (Alwathnani, 2012), these are all accepted as successful antifungal agents. However, there are endophytic fungi which have failed to inhibit the growth of pathogenic fungi. This failure is probably due to the poor concentration of bioactive compounds in nonvolatile antifungal metabolites (Rahmansyah, 2013), and thus a higher level is critical for nonvolatile testing.

Not many discoveries have been made about the potential of endophytes for the production of antiviral compounds and very few compounds have been reported as antiviral agents (Das et al., 2018). Some prophylactic effects against human rhinoviruses have been reported for the endophytic fungus *Nigrospora* sp. isolated from *Bruguiera sexangula* mangrove taxon; metabolites isolated here are anthraquinones (Kjer, 2009). Moreover, altenusin obtained from *Alternaria* sp., an endophyte isolated from *Sonneratia alba*, also shows prophylactic effects against human rhinoviruses. Ding et al. (2010) reported that Xiamycin A obtained from *Streptomyces* sp. strain GT 2002/1503, a *Bruguiera gymnorrhiza* endophyte, shows selective anti-HIV activity. Two isoindolones from *Emericella* sp. endophyte (HKZJ), isolated from the inner bark of *A. corniculatum*, exhibit antiviral activity against influenza A virus (H1N1) (Zhang et al., 2011).

Malaria is a highly destructive human parasitic infection. More than 500 million people are affected and nearly 2.5 million people die every year. Four protozoan species of the genus *Plasmodium* (*Plasmodiun falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*) are responsible for this disease (Kaul et al., 2017; Mendis et al., 2009). Increasing resistance to the antimalarial drugs available currently stresses the need for investigation of novel drugs together with the treatment efforts to get rid of this deadly disease. According to de Silva et al. (2013) and Kaul et al. (2017) natural products could serve as potential sources for new antimalarial drugs because they contain a great variety of chemical structures which have been screened for antiplasmodial activity. Mangrove plants and their associated fauna, due to their richness in bioactive molecules, could be a good source in this connection, however very few investigations have been undertaken for their antimalarial potential (Das et al., 2018). Calcul et al. (2013) have published data on the presence of several metabolites in the endophytic fungus *Diaporthe* sp. from *A. marina*, *Kandelia obovata*, and *Lumnitzera racemosa* mangroves. They show antimalarial activity against *P. falciparum*, similarly butyrolactone V isolated from *Penicillium expansum* endophytic fungus from *E. agallocha* mangrove also shows antimalarial activity (Wang et al., 2012).

15.8.2 Anticancer and cytotoxic activities

Cancer affects more than 6 million people a year. Antitumor agents are the compounds which counteract the formation of malignant tumors. Plant-based compounds like taxol, vinblastine, vincristine, topotecan, and etoposide have played a great role in the development of several clinically useful anticancer drugs (Kaul et al., 2017; Nirmala, Samundeeswari, & Sankar, 2011). The paclitaxel extracted from the yew plant during the 1990s was reported to have activity against a broad band of tumors, including breast, ovarian, lung, head, and neck cancer (Nisa et al., 2015). Many endophytic fungi, such as *Chaetomell raphigera*, *Colletotrichum falcatum*, *Fusicoccum* sp., and *Pestalotiopsis neglecta* isolated from terrestrial medicinal plants also have been screened for the production of taxol (Gouda, Das, Sen, Shin, & Patra, 2016).

The chemo-, bio-, and ecodiversity of marine ecosystems has immensely contributed in the development of potent antitumor compounds (Das et al., 2018). A rich source of anticancer drug candidates can be obtained from marine organisms or their metabolites. According to Stierle et al. (1993) and Das et al. (2018), following the discovery of anticancer compound taxol from the endophytic fungus *Taxomyces andreanae*, the endophytes have been investigated at a dramatic scale for their anticancer potential. During the last 10 years, the number of preclinical anticancer lead compounds extracted from metabolites of marine-derived fungi has increased to greatly (Das et al., 2018).

In spite of all these efforts, there is a need for the exploration of alternative sources with more diversity and novelty. The unique secondary metabolites in the endophytes show tremendous diversity. Minimum side effects have been seen from the endophyte-derived active metabolites with anticancer action, and these compounds could be an alternative approach for the discovery of novel anticancer drugs (Chen, Fu, & Zhou, 2014; Kaul, Gupta, Ahmed, & Dhar, 2012; Kaul et al., 2017; Kharwar et al., 2011).

Studies have reported that taxol production is limited only to the medicinal plants, leaving other sources, mainly mangroves, uninvestigated (Gangadevi, Murugan, & Muthumary, 2008). However, Elavarasi, Rathna, and Kalaiselvam (2012) reported that the endophytic fungus *F. oxysporum* isolated from *Rhizophora malayana* was able to produce taxol. These studies and reports need to be further validated for the discovery of more potential sources of the taxol-producing organism in mangroves. As there is high demand for this compound, the mangroves may serve as a tremendous source for its production. A recent study carried out on the anticancer activity of marine fungus *Penicillium cyaneum* against Hepg2 cell line (human liver carcinoma cells) have shown potent cytotoxic effects with an IC_{50} value of 242 μg/mL for ethyl acetate fraction of the fungus (Fernandes, Mohamed Salique, & Umamagheswari, 2017).

Apart from volatile and nonvolatile metabolite production and antimicrobial activity, the mangrove endophytic fungus associated plant *K. candel* has been reported to possess cytotoxic activity (Liu et al., 2017). Twenty new metabolites and two known compounds have been successfully extracted from an endophytic fungus, *Pseudolagarobasidium acacilcola*, isolated from a mangrove tree, *Bruguiera gymnorrhiza* (Wibowo et al., 2016). The extracts have been tested for their cytotoxic activity, and their structures elucidated through spectroscopic data analysis. From the analysis, a compound that exhibited cytotoxic activity is terpene endoperoxide, while a compound without the endoperoxide moiety did not show the cytotoxic activity. This evidence suggests that endoperoxide moiety of sesquiterpene is a necessary functionality for the compound to exhibit cytotoxicity. Cytotoxicity of endophytic fungi is supported by several other studies emphasizing the influence of compounds with an endoperoxide moiety. The endoperoxides are also regarded as a good source of drug candidates for the treatment of malaria and cancer (Das, 2015). The cytotoxic activity of sesquiterpene endoperoxide probably originates from the reactive radical produced from a homolytic cleavage of endoperoxide, as proposed by Wibowo et al. (2016). Such radical species possibly react with individual biological molecules like enzymes or proteins which later get transformed into a compound that inhibits the cell growth. Therefore it will be of great interest to decipher the underlying mechanism of the reactions and know the biomolecules involved in the production of the compound. A recent study by Yang et al. (2017) screened the cytotoxic activity of a novel compound Aspergone against the growth panel of 10 tumor cell lines and found it to be a potent cytotoxic compound (Yang et al., 2017).

15.8.3 Therapeutic agents for Alzheimer's disease

A neurodegenerative disease of the central nervous system, Alzheimer's disease (AD) is a memory and movement dysfunction. Out of nearly 15% of elderly people with different degrees of dementia, approximately 60%−70% have AD. The pathogenesis of senile dementia is not clear (Kaul et al., 2017) but the most accepted hypothesis is cholinergic nerve injury. If this is true, for an effective improvement of this disease and treatment acetylcholinesterase inhibitors could be developed (Anand & Singh, 2013; Kaul et al., 2017).

Previous clinical–pathological investigations show that beta-amyloid (Aβ) peptide deposition in brain parenchyma and cerebral blood vessels are hallmarks of AD as these are the amyloid precursor protein (APP) fragments (Lefterov et al., 2010; Wu et al., 2014). Several studies conducted in the past have blamed Aβ fibrils for the AD, however, recent findings show a decisive correlation between the levels of soluble, nonfibrillar Aβ oligomers and the extent of synaptic loss and cognitive impairment (Wu et al., 2014). In comparison with Aβ fibrils and plaque, Aβ oligomers are indicated to be more potent as neurotoxins that cause disturbances in the neuronal synaptic plasticity (Broersen, Rousseau, & Schymkowitz, 2010; Wu et al., 2014). The relationship between Aβ peptides oligomerization, cellular dysfunction, and AD suggests that inhibition of oligomerization might open the doors for researchers to discover novel therapeutics compounds for the treatment of AD (Singh, Srivastav, Yadav, Srikrishna, & Perry, 2016; Wu et al., 2014).

The bioactive fraction ME0-W-F1 extracted from *Phomopsis occulta*, has been isolated from *Pongamia pinnata* mangrove, which can reduce the formation of high-molecular-weight (HMW) Aβ42 oligomer and tetramer in vitro. This will be possible by an inhibition in the formation of β-sheet secondary structure. Effect of *Phomopsis occulta* compounds on the Aβ42 aggregation has been evaluated using an *E. coli* model developed during previous studies (Fisher, Kim, & DeLisa, 2006). The *E. coli* model is also suggested where the fusion protein is mentioned in the presence of samples with Aβ42 aggregation activity. The protein can be exported to the extracellular space by degrading Amp. The growth rate of *E. coli* seems to be proportional to the inhibitory effect of samples on the Aβ42 aggregation (Wu et al., 2014).

The salinity levels of living environment affect the bioactivity of secondary metabolites from *P. occulta*. No robust growth-promoting effects have been observed with the bioactive fraction extracted from fungi grown at 1 M NaCl, which could be due to *P. occulta* growth conditions. The natural seawater contains nearly 0.75 M NaCl, but even 1 M NaCl is not excess stress for them. *P. occulta* mycelial extracts grown at 0, 2, and 3 M NaCl show strong growth-promoting effects in *E. coli* model, signifying the fact that under salinity stress conditions some of the secondary metabolites produced show antiaggregation activity (Wu et al., 2014). The α-glucosidase inhibitory effects of benzomalvin A from the fungus *Penicillium spathulatum* have been confirmed in vivo with an oral sucrose tolerance test in normal and hyperglycemic mice ($P < .05$) (Del Valle, Ana-Laura, Figueroa, Huzefa, & Rache, 2016).

15.8.4 Antidiabetic activity

The reports published by Cui et al. (2016), Kaul et al. (2017), and Ozturk et al. (2018) reveal that lately more than 400 million people in the world have been recorded as having diabetes, 90% of the cases being diagnosed as type II diabetes cases.

The bioactivity studies of secondary metabolites of mangrove endophytic actinomycetes show that they possess PTP1B inhibitor and glucosidase inhibitor characteristics. Several compounds derived from the endophytic fungus *Penicillium chermesinum*, isolated from *K. candel*, exhibit strong α-glucosidase inhibition activity (Huang et al., 2011). Uncharacterized compound 07H239- has been isolated from the endophytic fungus *Xylaria* sp. BL321 has been isolated from *A. ilicifolius* mangrove and shows inhibitory activity on α-glucosidase (Song et al., 2012). Again a vermistatin derivative HN29-3B1 isolated from the *Penicillium* sp. mangrove endophytic fungus *Cerbera manghas*, shows α-glucosidase inhibitory activity (Liu et al., 2014). Rubrofusarin B isolated from the endophyte *Aspergillus tubingensis* from *P. pinnata* also exhibits mild α-glucosidase inhibition activity (Huang et al., 2010).

15.8.5 Antioxidant activity

A comprehensive review of the antioxidant profile of endophytic fungi has been published at length by Finkel and Holbrook (2000) and Hamilton, Gundel, Helander, and Saikkonen (2012). In reducing the risk of these ROS-mediated diseases, antioxidants are believed to be highly effective as the radical scavengers (Li et al., 2015). Following the discovery of pestacin and isopestacin as antioxidant compounds from endophyte *Pestalotiopsis microspora* living inside *Terminalia morobensis*, research on the antioxidant compounds from endophytes has gained much importance (Harper et al., 2003). Kaul et al. (2017) have reported data related to the isolation of diverse antioxidant compounds.

Antioxidant activity of *Trichoderma* sp., an endophytic fungus isolated from 12 mangrove species, has been reported by Saravanakumar and Kathiresan (2014). The presence of phenols indicates antioxidant activity by removing toxic free radicals as bioactive compounds (Kasote, 2013; Rankovic, Kosanic, & Stanojkovic, 2011; Zafra-Rojas et al., 2013). There is a positive relationship between the total phenol content and antioxidant activity of individual

organisms (Liu & Ng, 2000). The relationship supported by a recent study (Saravanakumar & Kathiresan, 2014) stresses the fact that *Trichoderma* strain shows maximum antioxidant activity, due to high phenolic derivatives in them. Similarly, *Trichoderma* strain has also been tested with 2-diphenyl-1-picrylhydrazyl (DPPH) to identify the presence of potential antioxidant compounds, and the tested *Trichoderma* extract displayed high free radical scavenging activity (Zanwar, Hegde, & Bodhankar, 2010). From the observation of Saravanakumar and Kathiresan (2014), the higher the concentration of the extract, the greater was the hydroxyl scavenging activity exhibited by *Trichoderma*. Thus they concluded that the hydroxyl scavenging activity is dose dependent and increases with increase in concentration.

An analysis of the extract using gas chromatography−mass spectrometer (GC-MS) revealed several metabolites including pregnane-3, diacetate, methyl ester, oleic acids, and a few other metabolites, which are responsible for the high antioxidant activity shown by *Trichoderma* strain (Saravanakumar & Kathiresan, 2014). According to Al-Olayan et al. (2014), diacetate is reported to be a potential hepatoprotective compound. These findings have indicated that endophytic *Trichoderma* is a potential source of antioxidant compounds (Saravanakumar & Kathiresan, 2014). A study by Handayani, Rivai, Hutabarat, and Rasyid (2017) tested the antimicrobial activity of endophytic fungal strains *Sonneratia griffithii*; roots are more antimicrobial compared with leaves and bark and this fungal strain can be developed as a new source of antibiotic compounds (Handayani et al., 2017). Thus all the above studies acknowledge the role of endophytic fungi in the production of antibiotics with a great variety and on the other hand can target the MDR pathogens as well.

15.9 Production of enzymes

Endophytes are symbiotic microorganisms of living plants and potential sources of biologically active natural products including microbial enzymes (Gouda et al., 2016). Microbial enzymes are widely used in pharmaceuticals, food, textile, leather, fine chemicals, and other industries. Microbial proteases, as a source of enzymes commercially employed in various industries, are preferred due to their rapid growth, limited space needed for cultivation, and ease of genetic manipulation to generate new enzymes with desirable properties (Najafi, Deobagkar, & Deobagkar, 2005; Orlandelli et al., 2015). The potential endophytic fungi have been investigated with an aim to examine the extracellular enzymes production in liquid media. From this study, Gouda et al. (2016) have found that the endophytic fungi produce few enzymes including urease, amylase, cellulase, pectinase, and chitinase. Earlier studies have proven that *Eutypella*, a mangrove endophytic fungi, has been found to be a promising source of many exoenzymes including cellulases, proteases, laccases, lipases, and amylases (Willsey & Wargo, 2015). The rhizo- and endophytic bacteria have been isolated from soil, leaves, and roots by using different enzymatic culture media. A total of 46 rhizophytic and endophytic bacteria showing various enzymatic activities have been isolated on 1/10 R2A media containing appropriate substrates (skim milk, tributyrin, and starch) (Bibi et al., 2017).

15.10 Heavy metal tolerant property

Endophytic fungi have been found to possess metal resistance. Choo, Sabri, Tan, Mujahid, and Müller (2015) isolated an endophytic fungus closely related to *Pestalotiopsis* sp. It has shown resistance to copper, chromium, lead, and zinc. A similar trend for endophytic fungi has been reported in several studies, with resistance to copper, zinc, and cadmium (Choo et al., 2015; Miransari, 2017). Even though all isolates were closely related to *Pestalotiopsis* sp., some of the isolates even matched to the same species, *Pestalotiopsis microspora*, but did not possess the equivalent tolerance to the heavy metals as *P. microspora* (Choo et al., 2015). The difference in heavy metal tolerance capacity is probably due to the uniqueness of the resistance mechanism strategy exhibited by the fungi (Iram et al., 2012). Congeevaram, Dhanarani, Park, Dexilin, and Thamaraiselvi (2007) reported that there are fungal species which show greater resistance to a high concentration of chromium (10,000 ppm). Fungal strains from two studies (Congeevaram et al., 2007; Faryal, Tahir, & Hameed, 2007) were isolated from a heavy metal-contaminated environment. The environmental pressure on the microorganisms plays a critical role in the adaptation and resistance of the endophytic fungi to the persisting environment (Choo et al., 2015).

A substantial reduction of the copper ion was reported among plants treated with *Purpureocillium* sp. A5, which exemplifies a buoyant potential use for environmental remediation using endophytes. It may also elucidate partly the enormous capacity of mangrove ecosystems in capturing heavy metals (Gong, Liu, Liao, Song, & Zhang, 2017).

15.11 Biocontrol agent

The application of chemical insecticides is accepted now as being of great concern to health and environmental issues due to their disadvantages. This has subsequently led to the innovative alternative measures including biological control or bioinsecticides (Mnzava et al., 2015). Biological control using microorganisms that are capable of inhibiting or antagonizing plant pathogens and pests can replace the present chemical pesticides which are having various side effects (Orlandelli et al., 2015). Fungal endophytes are known as effective antagonists (Azevedo, Maccheroni, Pereira, & de Araújo, 2000). Endophyte activities against phytopathogens and pests have been verified by various in vitro and in vivo experiments and have proven that endophytes could be effectively used as biological control agents (Costa et al., 2013). Endophytes compete with phytopathogenic fungi through the production of hydrolytic enzymes, such as proteases and chitinases, which are capable of degrading the hyphal cell walls of pathogenic microorganisms (Orlandelli et al., 2015). This phenomenon was proven when the endophytic fungi, *Alternaria destruens*, that was isolated from various host plants including mangroves, showed herbicidal activity synergistically with other herbicides against *Cuscuta* (Dodder plant), a parasitic weed (Cook, 2006).

In a study conducted by Mnzava et al. (2015), the crude ethyl acetate extracted from mangrove fungal endophyte was evaluated for its toxicity. The brine shrimp (*Artemia salina*) test was used here for the preliminary screening. Extracts which exhibited the highest toxicity were further examined for insecticidal activity against *Spodoptera litura* larvae and acetylcholinesterase (AChE) activity. According to Harwig and Scott (1971), extracts that can cause larval mortality greater than 90% are considered as highly toxic. More than 100 fungal endophyte extracts have been screened; among them, only five extracts have displayed the highest toxicity at a concentration of 125 ppm (Mnzava et al., 2015). The five extracts were identified as *Aspergillus oryzae*, *Aspergillus tamarii*, *Aspergillus versicolor*, and two strains of *Emericella nidulans*. From the brine shrimp lethality test, it was concluded that the degree of toxicity increased with the concentration rise. An application of these five extracts on *S. litura* III instar larvae to observe their toxicity reaction revealed that *A. versicolor* shows the highest mortality rate (43.3%), even higher than positive control deltamethrin, a commercial insecticide (36.7%). The application of the bioassay method described earlier has illustrated that *Aspergillus* exhibited insecticidal activities against *S. litura* larvae. The 4-(*N*-methyl-*N*-phenyl amino)-butan-2-one compound produced by *Aspergillus gorakhpurensis*, an endophytic fungus, yielded about 50% mortality rate when applied to *S. litura* IV instar larvae. The results of the topical application bioassay indicated that all the five extracts tested had acute toxicity toward *S. litura* larvae. The degree of acute toxicity reported for the five extracts are higher in this study compared to previous studies. These findings reveal that these five extracts may become a resilient source of bioactive compounds for use as bioinsecticides (Abraham, Basukriadi, Pawiroharsono, & Sjamsuridzal, 2015; Mnzava et al., 2015).

Various *Streptomyces* sp. are capable of acting as biocontrol agents, both under laboratory and greenhouse/growth chamber conditions (Law et al., 2017). Studies have revealed that *Streptomyces* exhibits inhibitory activity against *Magnaporthe oryzae*. A decrease by up to 88.3% has been reported in rice blast disease in greenhouse studies if infected rice seedlings are treated with *Streptomyces*. The *Streptomyces* sp. therefore can be used as very effective biocontrol agents against rice blast disease (Law et al., 2017).

Conclusions

Extensive research has been carried out to discover more insights into endophytic fungi and the application of their secondary metabolites. All agree that endophytic fungi have the potential to produce novel bioactive metabolites including antimicrobial compounds, extracellular enzymes, antifungal compounds, antioxidants, and anticancer compounds. Among the natural product-based drugs, drugs from the microbial community have a significant contribution. Thus the exploration of the natural products from mangrove endophytic fungi should be enhanced to solve various problems faced by current antibiotics.

All these reports elucidate the fact that increasing our knowledge about endophytic fungi will open several probabilities for researchers to discover more novel bioactive secondary metabolites. Biotransformation methods can be applied to produce compounds that have no side effects on humans, plants, and environment. We hope that in the near future the structures of these known metabolites will be modified as per the need to enhance the bioactivity of the endophytic fungi, and improve the efficacy and specificity to the target microbes. The conditions of endophyte fermentations could be optimized in enhancing the metabolite productions by the endophytes. A broad knowledge of various diseases at the gene level may assist in the development of target-based drugs from the bioactive compounds as a whole. Next-generation sequencing technologies can achieve this by an in-depth study of protein targets and the bioactive compounds interactions.

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Chapter 16

Essential oil of mint: current understanding and future prospects

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16.1 Introduction

Medicinal plants have been used as a potent tool in overcoming various disorders, infections, and diseases since the beginning of the life on Earth. They play an important role in providing health benefits to a large number of people especially the rural population who reside in far-flung areas of the developing countries. According to the report published in The Wealth of India (1992), herbal medicines are suitable for primary health care. They may be used directly as therapeutic agents, dyes, organic compounds for the synthesis of drugs, teas, or for their natural chemical constituents. Approximately 70,000 plant species have been used for medicinal purposes at different times in different countries. Plants such as mint, garlic, turmeric, fennel, etc. are directly used as a medicine by a number of countries around the world (Table 16.1). Plant metabolites include primary metabolites and secondary metabolites, which depending on their composition include alkaloids, glycosides, corticosteroids, and essential oils. Due to the important uses of the essential oil of mint, efforts must be made to increase the oil content of this plant.

Mint (*Mentha*) is an important plant used extensively for medicinal purposes. It belongs to the family Lamiaceae, commonly known as the mint or dead nettle family. Its distribution is influenced by biotic and abiotic factors (Ozturk and Gork, 1978a, 1978b, 1979a, 1979b, 1979c, 1979d). In the mint family, *Mentha arvensis* is among the most important medicinal plants. *M. arvensis* is a highly aromatic medicinal plant owing to its essential oil composition. The essential oil yield of mint has been increased by the action of radiation-processed polysaccharides which are broken down into their monomeric or oligomeric form to act as an elicitor for the crops (Fazili, Wani, & Bhat, 2017). To cope with the needs for the high drug demands the medicinal plants are to be grown on commercial scales (Qureshi, Israr, Abdin, & Iqbal, 2005). The improvements in agronomical techniques have proved beneficial in enhancing the quality and yield of mint, which in turn enhances the production of secondary metabolites in this medicinal and aromatic plants

TABLE 16.1 Major essential oils of the world used for domestic purposes.

Mentha piperita	Cedarwood (Chinese)
Cornmint (*Mentha arvensis*)	Eucalyptus
Spearmint (*Mentha spicata*)	Orange
Sassafras (Chinese)	Coriander
Sassafras (Brazil)	Grapefruit
Khas	Lemon
Patchouli	Camphor
Lavander	*Litsea cubeba*

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(Fazili et al., 2017; Ozturk, Secmen, & Pirdal, 1986; Sabharwal, 2004). The present review provides comprehensive details on the use of mint as a medicinal plant, and the research prospects to enhance oil productivity of the plant.

16.2 Mint cultivation

Usually a tropical climate is not suitable for the cultivation of mint. However Japanese mint can be cultivated in both tropical as well as subtropical areas with average temperature ranges of 20°C−40°C with annual rainfall of 100−110 cm. Mint can be cultivated in wide variety of soils, however, the best soils required for ITS growth are sandy soils and loam soils. Studies have also been carried out on the soil samples from the fields with different soil characteristics, in particular regarding their micronutrient contents, and their effects on *M. arvensis* crop have been evaluated with regards to the herb, essential oil and menthol yields (Srivastava et al., 2002). The mint should be crop rotated with other crops in order to stop or reduce weed growth, for example, mint: potatoes, mint: rice, mint: peas etc.

Mints are very familiar and easily grown in many countries like Europe, Asia, North and South America, as well as Africa. The economically important *Mentha* taxa are cultivated extensively in several countries under the names peppermint, native spearmint, cornmint, and spearmint.

The demand for essential oils has increased in the United States, United Kingdom, France, and Germany, along with some eastern countries such as Japan, Hong Kong, and Singapore. The growth rate of essential oil trade normally is of 9% and 25% for domestic and export markets, respectively (Table 16.2). In India, the supply gap is about 12,000 tons (Weiss, 1997).

16.2.1 Disease and pest control management in mint

Mints are susceptible to multiple diseases, just like many agricultural crops, imposing significant production constraints affecting the yield as well as the quality of mint oils. These include fungal, nematode, viral or phytoplasmal, and bacterial diseases. Most serious diseases are caused by *Puccinia menthae*, *Alternaria alternate*, *Verticillium dahliae*, *Phoma stasserti*, *Rhizoctonia solani/bataticola*, and *Erysiphe cichoracearum*. The control of disease in mint is an important aspect for the removal of unwanted foreign agents/insect pests which infect the crop productivity drastically worldwide. Some of the important diseases and their causal agents are listed in Tables 16.3 and 16.4.

TABLE 16.2 Global production of essential oils: an overview.

Types of essential oil	300
Cultivation	Only 50% cultivated (rest harvested from wild plants)
Total production	120,000 tons
Estimated cost	Rs. 16,000 crores
Fragrance industry	Nearly 95% of EOs are used in the fragrance industry
Largest market	China > France > Germany > Italy > Japan > Spain > United Kingdom > United States

TABLE 16.3 Some important diseases of mint and their causal organisms.

Disease	Pathogen	Host	References
Downy mildew	*Pernospora stigmaticola*	*M. arvensis*	Cheng and Bai (1986)
White mold/stem rot	*Sclerotinia sclerotorium*	*M. piperita*	Faulkner (1962)
Typhula root rot	*Typhula itoanac*	*M. piperita*	Steenland and Burke (1950)
Stolon rot	*Macrophomina phaseoli*	*M. arvensis*	Bai, Chen, and Wang (1991)
Root rot	*Thielavia basicola*	*M. arvensis*	Cheng and Bai (1986)

TABLE 16.4 List of some pest/disease control management for mint.

Insect pests	Control and management
Cut worms	**Soil + Phorate (10 g)**
Caterpillar	**Melathion or Thiodan @ 1.5 mL/1 L of H_20**
Red pumpkin beetle	**Melathion @ 1 mL/1 L of H_20**
Mint leaf roller	**Weekly sprays of Thiodan @ 1.5 mL/1 L of H_20**
Disease	Control and management
Stolon rot	Crop rotation, treat the stolons with 0.25% solution of Captan or 0.30% Agallol solution or 0.10% of Benlate solution
Fusarium wilt	Solution of Bavistin, Topsin, or Benlate
Leaf blight	Copper fungicide

16.3 Mint oil

Secondary metabolites with structural diversity form the backbone of aromatic plants that have a great importance for food, cosmetics, and the pharmaceutical industry. The secondary metabolites like cyanogenic glycosides, terpenes, saponins, glucosinolates, tannins, polyacetylenes, and anthraquinones have a major role in the overall growth and development of crops. Essential oils are highly variable mixtures of terpenoids and aromatic compounds. Monoterpenes form their major component (Seigler, 1998) and the main source may be all vegetative organs. Several environmental factors and cultivation methods affect the lability of constituents of essential oil of mint.

16.3.1 Role of glandular trichomes in synthesis of essential oil of mint

Being lipophilic in nature, essential oils emerge from specialized secreting tissues called glandular trichomes. It was observed by Fahn (1979) and Çoban and Baydar (2017) that glandular trichomes play an important role in the synthesis of EOs, especially in *Lamiaceae*. However, in the case of peppermint, the synthesis of terpene is carried out by the secretory cells of glandular trichomes found on leaf and stem surfaces (Amelunxen, 1964; Fahn, 1979; McCaskill, Gershenzon, & Croteau, 1992). Scanning electron microscopic studies have been undertaken to estimate the gland numbers along with densities on developing leaves of *M. lavanduliodora* and peppermint (Maffei, Chialva, & Sacco, 1989; Maffei, Gallino, & Sacco, 1986). In general young leaves possess fewer glandular trichomes than older ones. Considerable variation has been found in monoterpene content between individual trichomes in peppermint (Turner, Gershenzon, & Croteau, 2000). The trend for menthol and related isomer production correlates with leaf size and age (Turner et al., 2000).

Hefendhel and Murray (1972) studied the biosynthesis of chemical constituents of various species of mint. The single Mendelian gene(s) is responsible for controlling the presence or absence of carvone, menthone, menthol, and piperitone or piperitenone. *M. arvensis* and *M. piperita* have been observed to perform almost the same conversions (Hefendhel and Murray, 1972). For the synthesis of terpenes geranylbisphosphate synthase, limonene synthase, cytP450 limonene 3-hydroxylase, *trans*-isopiperitenol dehydrogenase, *iso*-piperitone reductase, *cis*-isopulegone isomerase, pulegone reductase, and menthone reductase are the key enzymes involved (Çoban and Baydar, 2017; Croteau and Gershenzon, 1994; McConkey, Gershenzon, & Croteau, 2000).

16.4 Uses of menthol

The main component of mint oil is menthol which forms nearly 30%−40% of crude essential oil derived from mint species. The most widespread use of menthol is in the medicinal industry. It is used for medicinal purposes like inhalers, ointments, flavoring agents, pain balms, cough syrups, tablets, pharmaceuticals, dyes, and other medicinal usages. In addition, it is also widely used in food products, cosmetics, dental preparations, mouthwashes, soaps, and alcoholic liquors (Sujana, Sridhar, Josthna, & Naidu, 2013).

16.4.1 A source of biofuel

Biofuels are made from cellulosic biomass and a part of municipal solid and industrial waste. Plants act as good sources of biofuels, especially for neutralizing the carbon balance. However, a big limitation has emerged to reduce the growth of biofuel crops (Hood, 2016; Ozturk et al., 2017). They require farmlands which can be used for food crops. However, the technology to produce ethanol and biodiesel from plant biomass has made phenomenal progress (Hood, 2016; Ozturk et al., 2017). This has created a confidence that such an industry will be profitable and sustainable for future generations (Hood, 2016). The variations occur in the biomass, chemical composition, and structure (Kim and Dale, 2015a, 2015b). Kumar and Ramesh Bapu (2018) have used lemongrass and mint oil to reducing the emission of carbon monoxide, nitrogen oxides, hydrocarbons, and carbon dioxide, thereby enhancing the performance features.

16.4.2 Antioxidant and antiinflammatory features

The role of antioxidants in life is overwhelming (Seneviratne and Kotuwegedara, 2009). Herbs, spices, and aromatic plants are known as target plants for antioxidants. Plant leaves, such as mint, may contain active constituents which can regulate antioxidant, anticarcinogenic, and antimicrobial properties (Al-Saikhan, Howard, & Miller, 1995; Basanta et al., 2018; Bassiouny, Hassanien, Abd El-Razik Ali, & El-Kayati,1990; Dillard and German,2000; Reddy, Urooj, & Kumar,2005; Vinson, Hao, & Zubik, 1998).

Antiinflammatory effects of monoterpenes are not well-known. Some preclinical in vitro investigations have been undertaken to look at the potential role of L-menthol and mint oil as an antiinflammatory drug (Juergens, Stöber, & Vetter, 1998); for this purpose LPS-stimulated monocytes from healthy volunteers were used (Gupta, Pacheco, & Prakash,2018). L-Menthol has proved more antiinflammatory compared to mint oil at therapeutically relevant concentrations (Juergens et al., 1998). The clinical trial analysis has revealed that L-menthol has potential for therapeutic efficacy in bronchial asthma, colitis, and allergic rhinitis (Juergens et al., 1998). *Mentha* extracts contain constituents with anti-inflammatory effects (Table 16.5). The antiinflammatory activity of peppermint essential oil under in vitro conditions has been determined by 5-lipoxygenase inhibition assay (Brahmi, Khodir, Mohamed, & Pierre, 2017) and an effective inhibition of nitric oxide and prostaglandin E2 production in lipopolysaccharide activated RAW 264.7 macrophages (Brahmi et al., 2017; Sun, Wang, Wang, Zhou, & Yang, 2014; Tsai et al., 2013).

Hussain, Anwar, Poonam, Ashraf, and Gilani (2010) and Sun et al. (2014) have observed that the essential oils from *M. piperita* leaves grown in China have shown antiinflammatory activities in a croton oil-induced mouse ear edema model.

16.4.3 Antibacterial, antimicrobial, and cytotoxic activities

The extracts from the root, stem, and leaf of *M. piperita* have been evaluated by Sujana et al. (2013) for their antibacterial potential against *Streptococcus pneumonia*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Klebsiella pneumonia* following the agar well diffusion method. The extracts of leaves have strong antibacterial activity against a range of pathogenic bacteria (Sujana et al., 2013). *M. piperita* ethyl acetate leaf extract proved more inhibitory than chloroform, hexane, and petroleum ether.

Hu et al. (2005) studied the antibacterial activity of lyase depolymerized alginate against 19 bacterial strains. They received a series of mannuronic acid (M-block) and guluronic acid (G-block) fraction by lyase depolymerization of alginate (Hu et al., 2005). The fraction of mannuronic acid (M-block) and guluronic acid (G-block) shows antibacterial activity, however, the M-block fraction shows broader spectrum and more potent inhibition than G-block fractions (Cao et al., 2011; Hu et al., 2005). Hussain et al. (2010) have investigated the antimicrobial activity of *Mentha* essential oils against several microorganisms, using broth microdilution susceptibility and disc diffusion assays. Essential oils have been tested for their cytotoxicity on breast (MCF7) and prostate cancer (LNCaP) cell lines. MTT assays have been used to test the antimicrobial and cytotoxic activities of essential oils, but *M. arvensis* has proved relatively better compared to *M. piperita*, *M. longifolia*, and *Mentha spicata* (Hussain et al., 2010).

16.5 Elicitors in mint production: a case study

Plant growth, development, and yield, as well as essential oil quality, are improved with the application of growth regulators. Essential oils possess two or three major components in relatively high concentrations (20%−95%). These are menthol (59%) and menthone (19%) in *M. piperita* oil (Yalçınöz and Erçelebi, 2018).

TABLE 16.5 Health indications of *Mentha spicata, M. piperita,* and *M. arvensis*.

Health indications	References
Mentha spicata	
Leaf and stem infusion for headache and tiredness	El-Hilaly, Hmammouchi, and Lyoussi (2003)
Used in the expulsion of parasitic worms, especially *Ascaris lumbricoides*	Di Stasi et al. (2002)
Acts as a stimulant, carminative uses, headaches, antispasmodic, fever, the boiled leaves extract is used to relieving hiccups, flatulence, antiinflammatory, bronchitis, to prevent vomiting during pregnancy	Kumar and Chattopadhyay (2007)
Used in treatment of loss of appetite, common cold, asthma, bronchitis, sinusitis, fever, nausea, and vomiting	Kunnumakkara, Chung, Koca, and Dey (2009)
Used in viral hepatitis, acts as analgesic, memory enhancer, fever, bronchitis and are used as lotion in aphthae, as stomachic and diuretic, for gas pain, rheumatism, toothache, muscle pain, and mouthwash	Arumugam, Gayatri Priya, Subathra, and Ramesh (2008)
Mentha piperita	
Peppermint oil has been used traditionally as an antispasmodic (gastrointestinal chamber and bile ducts) and to cure irritable bowel syndrome, catarrh of the human respiratory tract, inflammation of the oral mucosa. Extensive use in myalgia and neuralgia, relaxation of menstrual cramps chicken pox, migraines	Balakrishnan (2015)
Peppermint plants have been used for many conditions, including loss of appetite, common cold, bronchitis, sinusitis, fever, nausea, vomiting, and indigestion	Kunnumakkara et al. (2009)
Peppermint uses include irritable bowel syndrome, flatulence, indigestion, nausea, vomiting, cough, and bronchitis, reduce fatigue	Lv et al. (2012)
Mentha arvensis	
Leaf and stem infusion for headache and tiredness	El-Hilaly et al. (2003)
Possesses abortifacient property	Kunnumakkara et al. (2009)
Acts as a stimulant, carminative uses, headaches, antispasmodic, fever, the boiled leaves extract is used to relieving hiccup, flatulence, antiinflammatory, bronchitis, to prevent vomiting during pregnancy	Kumar and Chattopadhyay (2007)
Used in treatment of loss of appetite, common cold, asthma, bronchitis, sinusitis, fever, nausea, and vomiting	Kunnumakkara et al. (2009)
Used in viral hepatitis, acts as analgesic, memory enhancer, fever, bronchitis and are used as lotion in aphthae, as stomachic and diuretic, for gas pain, rheumatism, toothache, muscle pain, and mouthwash	Arumugam et al. (2008)

16.5.1 Sodium alginate

Among the natural polysaccharides, alginates, which originate from *Sargassum*, occupy a prominent position (Anthony et al., 2007). Sodium alginate (SA) has homopolymeric poly-(1,4) D-mannuronic acid residues and poly-(1,4) L-gluronic acid residues (Idrees et al., 2011a). Being multifunctional in nature, it is a marine bioactive material. Gamma ray irradiations degrade and bring down the high molecular weight of some natural polysaccharides like alginates, carrageenan, and chitosan into smaller sized oligomers (Idrees et al., 2011a).

The oligomeric form of sodium alginate when applied to the agricultural crops as foliar spray produces some morphophysiological activities, such as plant growth inclination, elongation of root/shoot, flower/fruit production, germination of seeds, suppression of heavy metal stress, and reduction of harvesting periods for several crops, which in turn reduce the use of pesticides and insecticides (Hien et al., 2000).

Irradiated SA has been applied as 20, 40, 60, 80, 100, and 120 mg/L to determine the best foliar concentration for the best performance in terms of different growth parameters, together with herbage yield per plant, as well as physiological and biochemical parameters, such as total chlorophyll content, total carotenoid content, nitrate reductase activity,

TABLE 16.6 Volatile oil composition of *Mentha spicata* (var. MSS-5).

S. no.	Retention time	Constituents	S. no.	Retention time	Constituents
1	2.310	α-Pinene	16	14.777	Borneol
2	2.408	*cis*-Limonene oxide	17	15.016	Camphene
3	3.567	*trans*-Limonene oxide	18	16.124	Sabinene
4	3.882	Myrcene	19	18.135	Caryophyllene oxide
5	3.905	β-Pinene	20	18.412	Germacrene D
6	5.256	*p*-Cymene	21	21.345	Germacrene A
7	5.454	3-Octanol	22	21.487	Spathulenol
8	5.671	Limonene	23	22.987	Isobornyl acetate
9	6.470	(Z)-β-Ocimene	24	23.310	*iso*-Dihydrocarveol acetate
10	6.982	1,8-Cineole	25	23.904	β-Bourbonene
11	7.083	Linalool	26	24.925	Monoterpene hydrocarbons
12	13.505	Pulegone	27	25.103	Oxygenated monoterpenes
13	13.682	Carvone	28	25.456	Sesquiterpene hydrocarbons
14	13.939	*cis*-Carveol	29	25.771	Spathulenol
15	14.165	*trans*-Carveol	30	28.483	Others

carbonic anhydrase activity and yield, and quality attributes like essential oil content and essential oil yield of *M. spicata* at 120 DAP (Çoban and Baydar, 2017; Idrees, Naeem, Aftab, & Khan, 2011b; Idrees et al. 2012). The foliar application of irradiated SA was significant for all parameters studied. In terms of growth parameters, there were increases in plant height (79.83%), fresh weight (49.11%), dry weight (58.00%), leaf area (22.71%), leaf yield (105.97%), and herbage yield (45.24%) over the control at ISA_{60} (60 mg/L) after 120 DAP. Biochemical parameters also showed an increase, that is, total chlorophyll content (24.32%), total carotenoid content (22.50%), nitrate reductase activity (26.54%), carbonic anhydrase activity (18.86%), and yield and quality attributes, such as essential oil content (up 31.70%) and essential oil yield (up 90.90%), over the control by the foliar application of ISA_{60} (60 mg/L) at 120 DAP. The active constituents of the essential oil of *M. spicata* were analyzed by GC/MS analysis. The volatile oil constituents of *M. spicata* and the GC-chromatograms obtained from *M. spicata* are given in Table 16.6.

16.5.2 Growth parameters

Cell division, cell enlargement, and differentiation in general exhibit the quality and quantity of plant growth and development. These are affected considerably by internal and external factors, together with the absorption and supply of nutrients. All these have considerable effects on cell metabolism as do the phytohormones/plant growth regulators (Aftab et al., 2014; Patel and Golakia, 1988; Taiz and Zeiger, 2006). The regulators modify the transcription, translation, and/or differential sensitivity of tissues (Aftab et al., 2014). Authors have used irradiated sodium alginate as a plant growth regulator. Its application has increased the plant height, fresh/dry weights, and leaf area/leaf yield/herbage yield per plant. Similar findings have been reported by Sarfaraz et al. (2011). According to his findings irradiated SA, applied as leaf sprays at concentrations of 20−120 ppm (Idrees et al., 2011a), has proved helpful in the improvement of growth, physiological/biochemical parameters, and yield/quality attributes of fennel significantly. Wallace and Wallace (2003) reported that more than 60 factors are responsible for limiting a crop. Furthermore, the role of alginate-derived oligosaccharides in root growth-promoting activity is well-known (Idrees et al., 2011a; Natsume, Kamao, Hirayan, & Adachi, 1994). Gamma-irradiated SA applied at concentrations of 20−100 ppm has promoted the productivity of tea, carrot, cabbage, rice, barley, and peanut (Aftab, Naeem, Idrees, Khan, & Varshney, 2013). SA when irradiated at a higher dose, resulted in increased dry matter of rice seedlings (Hien et al., 2000). The growth-promoting effects of degraded alginate have been reported by Tomoda, Umemura, and Adachi (1994) on barley roots. A foliar spray of

alginate-derived oligosaccharides have significantly promoted superoxide dismutase, catalase, and peroxidase enzyme activities in tomato seedlings, and have also reduced the damage caused by ROS induced under drought conditions (Idrees et al., 2012; Ruizhi et al., 2009). These findings show that alginate-derived oligosaccharides stimulate antioxidative enzyme synthesis in a short time, thus catabolizing ROS and thereby protecting the cell membranes from damage. Irradiated sodium alginate shows more growth-promoting activity and may act as a biofertilizer (El-Mohdy, 2017). The depolymerized alginate exhibited growth-promoting effects on the elongation of barley root, especially that of the radicle (Taiz and Zeiger, 2006). They observed the effective concentration of alginate (100−300 μg/mL) for elongation of roots with no inhibition at the highest concentration. Iwasaki and Matsubara (2000), using a mixture of oligosaccharides of SA, showed growth-promoting activity of SA at 200−300 μg/mL. With regard to the above fact findings, it was concluded that alginates act as an endogenous elicitor-like substance (Akimoto, Aoyagi, & Tanaka, 1999; Aoyagi, Sakamoto, Asada, & Tanaka, 1998; Natsume et al., 1994; Tanaka, Kaneko, Aoyagi, Yamamoto, & Fukunaga, 1996; Wichers, Malingre, & Huizing, 1983).

It is not yet known clearly if the receptors are located on the cell wall or whether the oligomers penetrate through the cell wall into the cell membrane or cytoplasm. However, it can be maintained that irradiated sodium alginate could trigger the cascading effect on the cell membrane in such a way that enzyme(s) may lead to increased plant physiological and metabolic processes, thus leading to improved growth and yield of the plants.

16.5.3 Physiological and biochemical parameters

The optimal concentration for *M. spicata* has proved to be 60 mg/L irradiated sodium alginate for the physiological/biochemical parameters, like total chlorophyll and total carotenoid contents, carbonic anhydrase activity, and nitrate reductase activity. The degradation of oligosaccharides promotes various physiological processes in different plants (Aftab, Naeem, Idrees, Khan, & Varshney, 2016). Therefore it may be conceived that application of irradiated SA may improve and stimulate the photosynthetic pigments, that is, chlorophyll and carotenoid content in this study. With the increase in dose rate from 100 to 120 mg/L irradiated sodium alginate, there was a gradual decrease in the chlorophyll and carotenoid content with each higher dose. The increase in chlorophyll content could be possibly correlated to the increased number and size of chloroplast, proper granule development, and the amount of chlorophyll per chloroplast (Dar, Uddin, Khan, Ali, & Varshney, 2016). In addition, the chloroplast formation leads to an increase in the lipid content of the leaves and chlorophyll constituents.

According to Lawlor, Boyle, Keys, Kendall, and Young (1988), Siddiqui, Khan, Mohammad, and Khan (2008), Aftab et al. (2014), Ali et al. (2014), and Ahmad, Jaleel, Shabbir, Khan, and Sadiq (2019) one of the most important zinc-containing proteins in crops is carbonic anhydrase, which plays an important physiological role in ion exchange, acid−base balance, carboxylation or decarboxylation, together with the diffusion of inorganic carbon between the cell and its environment and within the cell. The activity of carbonic anydrase plays a crucial role in CO_2 availability and photosynthesis (Coleman, 2000). It catalyzes the reversible hydration of CO_2 to carbonic acid, thereby increasing the CO_2 available to Rubisco (Dar et al., 2016). Its activity regulates the biosynthesis of carbon compounds showing locomotion to the sink organs. It is assumed that the effect of irradiated SA on carbonic anhydrase activity may be related to the number of underlying factors associated with irradiated SA-mediated stimuli as the cumulative carbon fixation activities of carboxylases and oxygenases are associated with the Rubisco. Studies regarding the role of Rubisco and PEP carboxylase have been done (Idrees et al., 2012; Tomoda et al., 1994). In addition, the significant effect of the application of irradiated SA on leaf carbonic anhydrase activity has been reported in beetroot periwinkle (Idrees et al., 2011a). The results obtained in the experiment are in accordance with those investigations done by Luan et al. (2003) showing the enhanced activity of enzymes and net photosynthetic rates in plants.

The initial stage of nitrate assimilation involves the reduction of nitrate to nitrite by the activity of nitrate reductase, which is a highly regulated enzyme (Huber, Bachmann, & Huber, 1996). The plants need an adequate requirement of nitrogen which is utilized in the biosynthesis of amino acids, nucleic acids, proteins, and other cellular constituents crucially important for overall growth, metabolism, and the development of plants. The rate-limiting enzyme nitrate reductase is directly or indirectly dependent on the metabolic sensors and/or signal transducers (Aftab et al., 2014), besides serving as an important key in determining plant growth and development. In this study a significant enhancement in nitrate reductase activity was achieved with 60 mg/L irradiated sodium alginate proving it to be the optimal dose for an increase in nitrate reductase activity in *M. spicata*. The possible reason for the increased nitrate reductase activity could be the increased uptake of nitrogen from soil that might have increased the nitrate concentration (Aftab et al., 2014). As nitrate reductase is an inducible enzyme, the substrate concentration essentially induces functional nitrate reductase via a nitrate sensing protein of an unknown nature and other regulator proteins involved in metabolic response (Campbell, 1999, 2002; Idrees et al., 2012; Scheible, Gonzales, Morcuende, Lauerer, & Geiger, 1997).

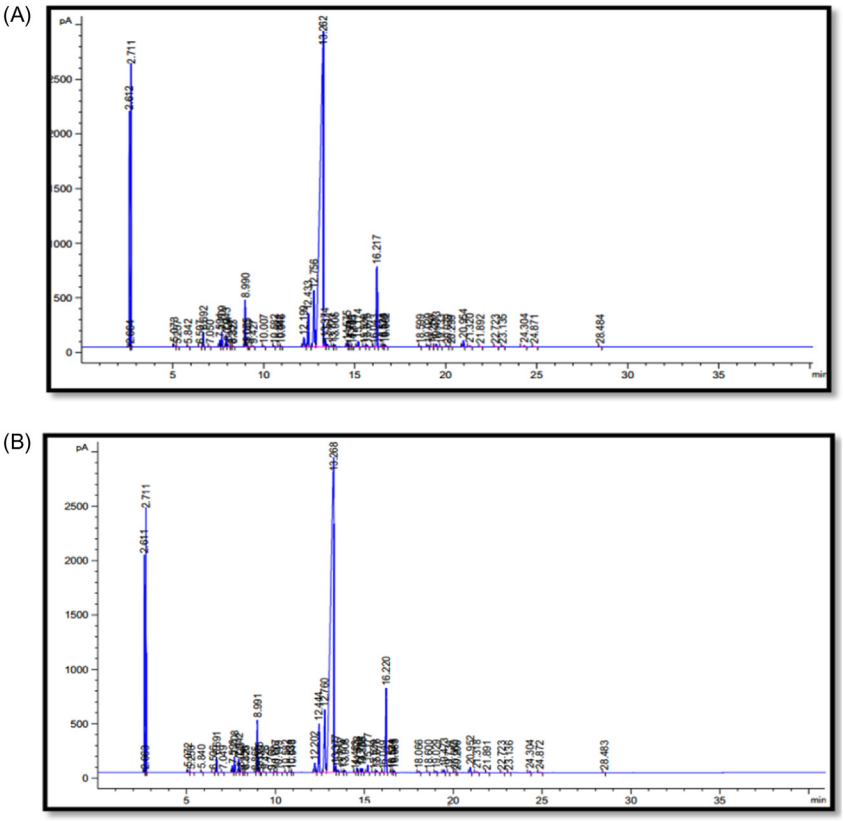

FIGURE 16.1 GC chromatogram of essential oil obtained from *Mentha spicata*: (A) at control and (B) at ISA_{60}. The increase in carvone content was recorded as 90.90% at ISA_{60} mg/L over the control.

16.5.4 Yield and quality parameters

Essential oil yield, essential oil content, and the yield of active constituents was taken into consideration. The accumulation of oil in plants depends mainly upon the developmental stage of the plant, such as the plant part, tissue, organ, or cells. The main life cycle of plants includes the origin of the primordia, their full maturity, and finally the process of senescence (deterioration with age) in which the leaves serve as a source of oil (Weiss, 1997). The application of irradiated SA has a positive effect on the yield and yield-attributing characters of *M. spicata*. Among the various doses of irradiated SA, 60 mg/L proved to be the best for the yield attributes in *M. spicata* as it improved the yield attributes by 31.70% and 75.00% (Fig. 16.1). The possible reason that there is better growth and yield of leaves may be correlated to the fact that the absorption of nutrients from the soil was increased in the treated plants, which in turn might have manifested in the improvement of the assimilation of translocation of photosynthates, which could have reciprocated the efficacy of growth, enhancement in physiology, and yield attributes, resulting in the greater oil yield and enhancement in the active constituents, with irradiated SA foliar application (Akimoto et al., 1999; Aoyagi et al., 1996; Idrees et al., 2012; Iwasaki and Matsubara, 2000; Kume, 2006; Luan et al., 2003). The yield and quality of essential oil in aromatic plants is also improved by irrigation and chemical fertilizers (Kapoor, Giri, & Mukerji, 2004; Tiwari and Banafar, 1995). This also supports the fact that irradiated SA application together with adequate nutrient supply enhances overall growth and yield. No record has been seen related to the effect of irradiated SA as a plant growth regulator on the content and yield of the essential oil of medicinal crops (Idrees et al., 2012). The findings of the effect of application of irradiated SA on essential oil content and yield are reported for the first time on *Mentha* sp. However, many other plant growth regulators have been used for this purpose (El-Keltawi and Croteau, 1986; Idrees et al., 2012; Misra and Srivastava, 1991).

Conclusions

The conservation, cultivation, and processing of aromatic and medicinal plants is of great interest for growing populations with respect to India. The 196 countries, including India, who are signatories of the Convention on Biological Biodiversity (CBD) have highlighted their demands for the conservation of aromatic plants including mint.

From the above results as well as the reviews studied, it is evident that the molecular as well as other mechanisms which actually enhance the productivity and yield of the medicinal plants could be the subject of research of great interest for future studies.

In addition, the exact mechanism underlying how the sodium alginate acts as a plant growth promoter is still unclear. Therefore in particular, the pathway of oil synthesis in *Mentha* spp. when exposed to irradiated sodium alginate should be worked out in detail.

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Chapter 17

Azadirachta indica: the medicinal properties of the global problems-solving tree

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17.1 Introduction

The neem tree, or the reliever of sickness, which is also known by its botanical name *Azadirachta indica* A. Juss., is a fast growing, evergreen tree that belongs to the Meliaceae family. It has an average natural life of over 200 year and initiates fruiting after 3−5 years. The neem tree can grow in poor-nutrient dry lands, tolerates high temperature, but is vulnerable to extreme cold or freezing conditions (Biswas, Chattopadhyay, Banerjee, & Bandyopadhyay, 2002; Koul, Isman, & Ketkar, 1990). The neem tree is native to the Indio-Pakistan subcontinent. It has also been introduced to many parts of Africa from Somalia to Mauritania and from Nigeria to Ghana, to the Caribbean islands, to Central America, to South America, to Southeast Asia (including China and Malaysia), to Europe, to the United States, and to Australia. In Africa the neem tree is considered as a leading candidate for helping to control and halt the desertification or the spread of the Sahara desert southward (Kumar & Navaratnam, 2013). Neem extracts and products have been used traditionally to treat human diseases since prehistoric times. The oil is extracted from the tree's fruits and seeds and used for many medicinal applications. Many bioactive compounds have been isolated from the different parts of the neem tree such as leaves, seeds, fruits, barks, flowers, twigs, gum, and roots. The reported uses of the neem tree included the treatment of respiratory disorders, intestinal helminthiasis, skin infections, blood morbidity, itching, skin diseases, burning sensations, antisnake venom, pthisis, and digestive disorders. The extracts are also reported to be used as antiinflammatory, antipyretic, antihistaminic, antioxidant, antiulcer, antifertility agents, for the treatment of sexually transmitted diseases, and have the potential for use as a long-term female contraceptive. Other uses include as an antifungal, antimalarial, antibacterial, antiviral, and for the treatment of parasitic diseases, as well as being anticarcinogenic with cancer chemopreventive potential, as an analgesic, hepatoprotective, immunostimulant, and for its effect on the central nervous system (Asif, 2012; Atawodi & Atawodi, 2009).

The identification and isolation of neem constituents was initiated about eight decades ago in India. The presence of carotenoids, polysaccharides, proteins, calcium, sulfur, phosphorus, amino acids, fatty acids, and fibers has been reported. Many novel chemical compounds have also been isolated from the various parts of the tree. These include protoliminoids such as meliantriol, nimbocinone, nimolinone, azadirachtol, and azadirachnol. Liminoids, such as mahmoodin and azadirachtin; tetranortriterpenes, pentanortriterpenoids, hexanortriterpenoids; and nontriterpenoidal constituents such as hydrocarbons, fatty acids sterols, phenols, flavonoids, glycosides, and diterpenoids (Biswas et al., 2002; Boeke et al., 2004; Kumar & Navaratnam, 2013).

Of the many remedial, pharmacological, and medicinal uses of neem products, data on the toxicological effects of neem products on many animal models such as rats, mice, chicken, and monkeys have been collected and published. The reported toxicological activities include allergy, gastrointestinal spasm, lethargy, and hypothermia in human and animals. Hepatobiliary disease in albino has also been reported as a result of consumption of aqueous extracts of *A. indica*. Liver biopsy of a human infant has also been reported. Other toxicological effects include nausea, general

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discomfort, and hyperkeratosis of the stratum corneum. It was found that the nonaqueous portions of the extracts were much more toxic than the unprocessed materials or the aqueous portions and these extracts may be used as insecticides, to protect stored seeds for human consumption, based on the most recent safety assessments (Atawodi & Atawodi, 2009; Boeke et al., 2004; Nanduri et al., 2003).

Neem products also show larvicidal, acaricidal, nematicidal, and insecticidal effects, especially their methanolic extracts. Due to these activities neem products are used for agricultural pest control. The extracts of *A. indica* have also been used as insecticides, and as insect repellants, especially mosquitoes, by the smoke of the leaves. The molluscidal effect of neem products has been reported on many snail species (Atawodi & Atawodi, 2009; Boeke et al., 2004). The antidiabetic activity of *A. indica* products has also been evaluated using animal models. The antihyperglycemic effect of the plant was pronounced; however, its mechanism of action is not very clear (Boeke et al., 2004) (Fig. 17.1).

Herein, we have summarized the major bioactive components of *A. indica*, along with their applications to treat important life-threatening diseases.

17.2 Anticancer properties of *A. indica* (neem tree)

Cancer is a perplexing challenge for the researchers in the health field. Despite the huge efforts made so far, no dependable treatment for most types of cancer has been discovered yet (Paul, Prasad, & Sah, 2011). Cancer cells exhibit several characteristic traits, such as an excessive cell growth, reprogramming of energy metabolism leading to the uncontrolled proliferation, cell resistance to death, angiogenesis, metastasizing to distant sites, and immune response suppression against tumor cells. Various types of cancer cell adaptations along with their underlying mechanisms are key for their survival. In this scenario, the inhibition of one or a few pathways will not ensure their targeted elimination. Consequently, health care researchers are targeting these cells via several pathways to avoid unfavorable side effects and to minimize patients' distress. The use of ethnomedicine and certain plant-derived herbal products are in the limelight of researchers and medical practitioners thanks to their significant anticancer properties and negligible adverse side effects (Biswas et al., 2002; Hao, Kumar, Yadav, & Chandra, 2014).

17.2.1 Anticancer study by Kigodi and coworkers

In a study by Kigodi and coworkers a known nimbolide and a new limonoid, 28-deoxonimbolide (Fig. 17.2), were isolated from the leaves of *A. indica* of Tanzanian origin. Both compounds were subjected to in vitro studies to examine their cytotoxic effects against seven cancer cell lines, P-388 (lymphocytic leukemia), KB (carcinoma of the nasopharynx), HT-1080 (human fibrosar-coma), LU-1 (lung cancer), COL-2 (colon cancer), MEL-2 (melanoma), and BC-1 (breast cancer). On these assays, nimbolide (**1**) exhibited cytoxicity activities with IC_{50} values of 0.39, 0.41, 0.31, 0.42, 0.53, 0.25, and 0.065 μg/mL while 28-deoxonimbolide (**2**) showed IC_{50} values of 1.34, 1.81, 1.04, 0.84, 2.05, 1.30, and 0.66 μg/mL, respectively, against these cell lines.

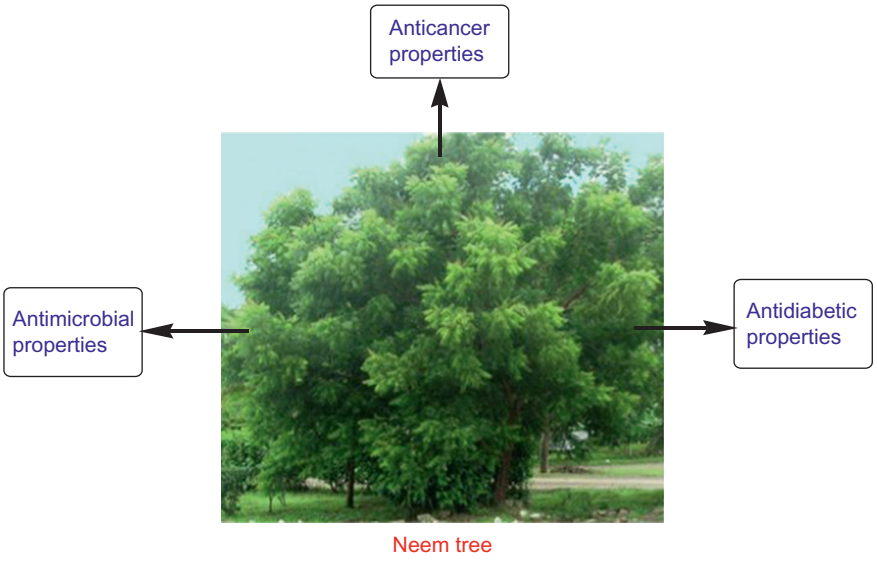
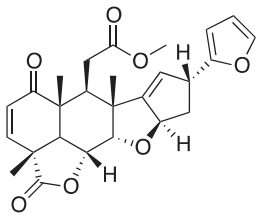


FIGURE 17.1 *Azadirachta indica* (neem tree) and its important medicinal applications.



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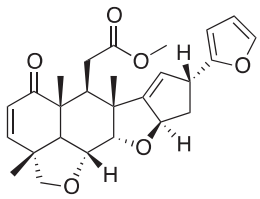

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FIGURE 17.2 Anticancer compounds identified by Kigodi and coworkers.

The anticancer activities of nimbolide and 28-deoxonimbolide against the various cell lines may be attributed to the presence of α-β-unsaturated ketone moieties. However, current data also suggested the ∂-lactone moiety may be considered as a potential contributor to the cytotoxicity of the abovementioned compounds (Kigodi, Blaskó, Thebtaranonth, Pezzuto, & Cordell, 1989).

17.2.2 Anticancer study by Kikuchi and coworkers

Various studies demonstrated that methanolic seed extracts of *A. indica* have strong anticancer activities against various cell lines. In a study by Kikuchi and coworkers the cytotoxic activities of 35 limonoids were investigated against five different human cancer cell lines, HL60 (leukemia), A-549 (lung), AZ521 (stomach), SK-BR-3 (breast), and CRL1579 (melanoma). These compounds were isolated from seed extracts of *A. indica*. Among these tested compounds, seven showed cytotoxicity activity against one or more than one cell line. These compounds included 7-benzoylnimbocinol (**3**), epoxyazadiradione (**4**), 7-deacetyl-7-benzoyl epoxyazadiradione (**5**), gedunin (**6**), 7-deacetyl-7-benzoylgedunin (**7**), 28-deoxonimbolide (**8**), and ohchinin acetate (**9**), as shown in Fig. 17.3. The compounds **5**, **7**, and **8** showed potent activity against HL60 leukemia cells with IC_{50} values of 3.1, 2.9, and 2.7 μM, respectively. Moreover, compound **5** showed potent activity against HL60 and AZ521 cells. Compound **7** showed potent activity against HL60 cells, while compound **8** exhibited potent activity against HL60, A-549, AZ521, and SK-BR-3 cells. These compounds altogether showed higher cytotoxicity activity than those of the reference compounds, cisplatin or 5-fluorouracil, used in the same assay.

The mechanism of action of compounds **5**, **7**, and **8** may be attributed to their ability to induce early apoptosis in HL60 cells. It was also observed that apoptotic cell death in these cases resulted from both the mitochondrial as well as the death receptor-mediated pathways. In addition, compound **5** exhibited high cytotoxicity selectivity for leukemia cells, proved by the very weak cytotoxicity against lymphocyte normal cell line (RPMI 1788).

Compounds **3**, **4**, **5**, **6**, **7**, **8**, and **9** showed promising anticancer activity with IC_{50} values ranging from 1.7 to 9.9 μM, against one or more types of cancer cell lines. While other compounds investigated in this assay showed moderate activity with IC_{50} values more than 20 μM. The structure−activity relationship revealed that azadiradione, gedunin, and nimbin types of limonoids acquire considerable cytotoxic activity, while azadirachtin-type and degraded limonoids have comparatively weaker cytotoxic activity (Kikuchi et al., 2011).

17.2.3 Anticancer study by Gualtieri and coworkers

As a matter of fact, all parts of the neem tree confer therapeutic use. Neem leaves have shown a wide range of health benefits. In a study by Gualtieri and coworkers 15 compounds, including eight new limonoids, one new phenol glycoside, and six previously known compounds, were isolated from the chloroform and methanol extracts of *A. indica* leaves. The compounds were evaluated for their cytotoxic effects against different cancer cell lines as well as their interactions with the molecular chaperone Hsp90.

In this investigation, the HeLa (human epithelioid cervix carcinoma) and PC-3 (human prostate adenocarcinoma) cancer cell lines were used to examine the cytotoxicity activity of 15 isolated compounds. Herein, the tested compounds did not show any significant cytotoxicity. However, some of compounds exhibited weak cytotoxicity activity with IC_{50} values in the range of 74 ± 6 to 95 ± 11 μM in HeLa and 95 ± 11 to 100 ± 00 μM in PC-3 cells, respectively.

Finally, all compounds were subjected to a surface plasmon resonance (SPR)-based binding assay to examine their interactions with Hsp90. Deacetylsalanin (**10**) and 1,3-diacetylvilasinin (**11**) (Fig. 17.4) showed greater affinities toward the chaperone which can be attributed to the presence of a functional group at C-1 and/or C-3 which affected their affinities for Hsp90 (Gualtieri et al., 2014).

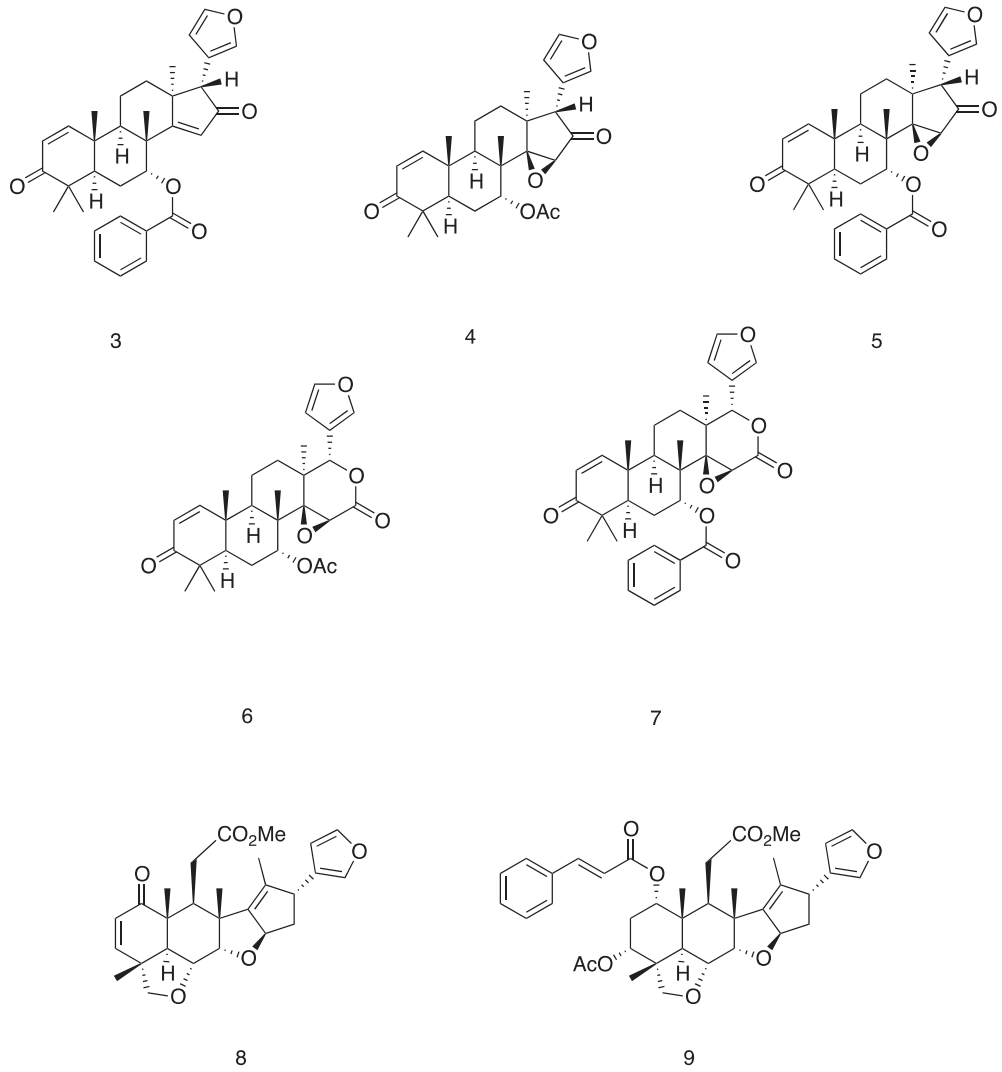

FIGURE 17.3 Anticancer compounds identified by Kikuchi and coworkers.

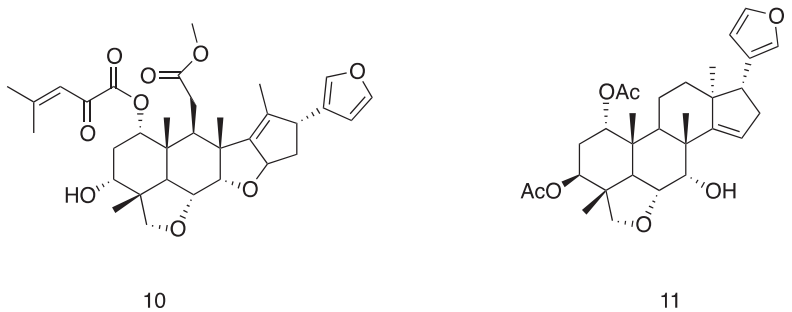

FIGURE 17.4 Anticancer compounds identified by Gualtieri and coworkers.

17.2.4 Anticancer study by Kashif and coworkers

Neem oil has a diverse chemical composition and biological activity depends on the soil nature and the climate conditions of the region in which it is nurtured. However, compounds like limonoids, glycerides, volatile compounds, terpenoids, and fatty substances are found in all neem oils. In a study by Kashif and coworkers the anticancer ability of

TABLE 17.1 Cytotoxicity IC_{50} values (μg/mL) of neem oil extract for different durations: 24, 48, 72, and 96 h.

Treatment duration	Cancer cell lines		Noncancerous cell lines		
	Du-145	PC-3	A-549	NIH3T3	CCD-18co
After 24 h	157.15	136.08	150.90	282.72	342.15
After 48 h	130.25	117.19	133.02	271.28	290.47
After 72 h	120.66	114.66	124.52	234.86	228.24
After 96 h	118.13	113.79	119.41	209.66	207.50

methanolic neem oil extracts was investigated. In this study, three cancerous cell lines including A-549, PC-3, and DU-145 and two noncancerous cell lines of NIH3T3 and CCD-18Co were employed to investigate the cytotoxic and apoptotic properties of neem oil extracts. PC-3 and Du-145 were used as human prostate cancer cell lines, A-549 as a human lung cancer cell line, CCD-18Co as a human colon fibroblast cell lines, and NIH3T3 as a mouse embryonic fibroblast cell line. Three different concentrations of neem oil extract, 31, 62, and 125 μg/mL, were used to treat the cancerous and noncancerous cell lines (Table 17.1).

These results revealed the ability of neem oil extract to significantly reduce the viability in all selected cancer cells treated with different concentrations of extract compared to the control in all cancer cell lines. Interestingly, the results show much less effect on noncancerous cell lines at low concentration. Moreover, a considerable increase in the amount of necrotic and apoptotic cells, and caspases 3, 8, and 9 activity, was observed in all extract-treated cancer cells compared to untreated cells, whereas no effect on noncancerous cell lines was observed. This study demonstrated that neem oil extract has a pronounced cytotoxic effect on cancer cells and exerted a lower concentration-induced apoptosis in cancer cells through the activation of caspases signaling pathways. The results gleaned from this research indicated that neem oil extract may contain one or more bioactive agent/s that can be used in future as a safe, effective, and efficient anticancer therapy (Kashif, Kim, & Kim, 2018).

17.3 Antidiabetic properties of *A. indica* (neem tree)

Diabetes mellitus (DM), characterized by abnormally higher blood glucose level than the normal (hyperglycemia), is one of the most rapidly spreading diseases of the present century (Abbas, Hussain, Hamaed, & Supuran, 2019). The World Health Organization (WHO) reported that diabetes is the seventh leading cause of death affecting up to 366 million people internationally with over 3 million deaths annually. The hyperglycemic condition is due to either a fault in insulin secretion, insulin action, or both (Kharroubi & Darwish, 2015; Verma, Gupta, Chaudhary, & Garg, 2017). Excess glucose in the blood adversely affects carbohydrate, protein, and fat metabolism, which lead to severe diabetic complications such as neuropathy, retinopathy, and nephropathy as well as cardiovascular problems (Abbas et al., 2016). Currently available hypoglycemic agents exhibited a number of adverse effects like gastrointestinal complaints, weight gain, headache, and hypotension (Tamrakar, Maurya, & Rai, 2014). Natural products with lesser side effects provide an excellent choice to complement current therapies for the treatment of diabetes mellitus. Plant-derived products are considered as effective therapeutic materials to target diabetes and their complications with the least side effects. Various studies have been done to evaluate neem extracts against diabetes in in vitro and in vivo animal models (Dallaqua et al., 2012; Gupta, Kataria, Gupta, Murganandan, & Yashroy, 2004). Previous studies showed that ethanolic leaf extract of neem considerably reduced the blood glucose index in alloxan-treated diabetic rats (Dholi, Raparla, Mankala, & Nagappan, 2011).

17.3.1 Antidiabetic study by Ponnusamy and coworkers

Multiple approaches have been considered to target both type-1 and type-2 diabetes mellitus. One such approach is the inhibition of human pancreatic α-amylase (HPA) enzyme to control postprandial hyperglycemic conditions after starch breakdown. Both porcine pancreatic α-amylase (PPA) and HPA enzymes share similar sequences, and structural and functional properties. In a study, Ponnusamy et al. isolated/semisynthesized nine limonoids from *A. indica* which were screened against α-amylase enzyme for their inhibitory potential. During this study, azadiradione (**12**), epoxyazadiradione (**13**), and gedunin (**14**) showed PPA inhibition activity with IC_{50} values of 138.4, 100.2, and 72.2 μM, respectively, while the other compounds were largely inactive.

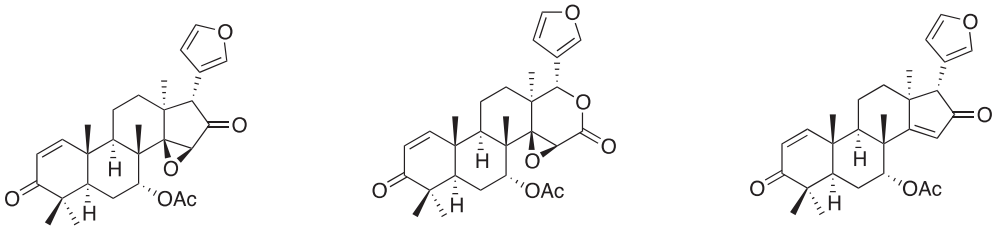

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FIGURE 17.5 Antidiabetic compounds identified by Ponnusamy and coworkers.

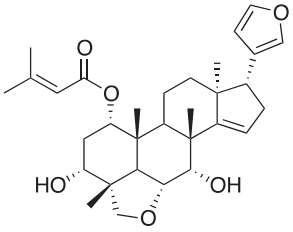

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FIGURE 17.6 Antidiabetic compound identified by Perez-Gutierrez and Damian-Guzman.

These compounds were then subjected to α-amylase inhibition activity to validate their hypoglycemic potential and mechanism of action. On HPA inhibition assay, azadiradione and gedunin (Fig. 17.5) exhibited significant inhibition in vitro with IC50 values of 74.17 and 68.38 μM, respectively. Acarbose was used as a standard inhibitor against HPA with IC50 value of 15 μM. Additionally, these compounds were investigated for their cytotoxic activities on AR42J α-amylase secretory cell lines where azadiradione and gedunin showed cytotoxicity with IC_{50} values of 11.1 and 13.4 μM, respectively. Structure−cytotoxicity relationship analysis revealed that α-amylase enzyme inhibition activity of limonoids was specifically due to the presence of a basic limonoid skeleton while compounds possessing C-seco type structural design did not show any activity against HPA. The molecular docking simulation studies exposed possible interactions between the aromatic amino acids of enzyme and inhibitors. The antidiabetic activity of *A. indica* could be attributed to its ability to inhibit human pancreatic α-amylase to control the hyperglycemic state after the meal (Ponnusamy et al., 2015).

17.3.2 Antidiabetic study by Perez-Gutierrez and coworkers

In a study, Perez-Gutierrez and coworkers investigated a new tetranortriterpenoid called the meliacinolin isolated form from *A. indica* for hypoglycemic effects, insulin resistance, α-glucosidase and α-amylase inhibition potential in streptozotocin (STZ)-diabetic mice. The results acquired from this study revealed that meliacinolin (**15**) reduced blood glucose level via increased insulin secretion by modulating the pancreatic secretion. This study showed that meliacinolin (Fig. 17.6) significantly inhibited α-amylase in a concentration-dependent way with an IC_{50} value of 46.74 μg/mL, whereas acarbose had an IC_{50} value of 12.23 μg/mL. Against α-glucosidase enzyme it showed an inhibition with IC_{50} value of 32.18 μg/mL as compared with acarbose's IC_{50} value of 78.54 μg/mL. These findings established meliacinolin as an interesting and promising antidiabetic agent thanks to its multiple actions of hypoglycemic activity, insulin augmenting effect, intestinal α-glucosidase enzyme inhibition, and pancreatic α-amylase enzyme inhibition (Perez-Gutierrez & Damian-Guzman, 2012).

17.3.3 Antidiabetic study by Satyanarayana and coworkers

A. indica (neem) is an important medicinal plant, used often in Ayurveda to cure a large number of diseases, including diabetes mellitus. In a study, an aqueous leaf extract of *A. indica* was prepared to investigate its effect on insulin signaling molecules expression and glucose oxidation in targeted tissue in diabetic male rats. Diabetic rats treated with *A. indica* leaf extract exhibited lowered blood glucose level and insulin concentration in close proximity to control, while glucose tolerance was near normal. Furthermore, studies of *A. indica* leaf extract on insulin receptor (IR) protein

expression revealed that IR protein level in extract-treated rats was 1.6-fold higher than IR protein in diabetic rats. In addition, an insulin receptor substrate-1 protein studies revealed that tyrosine phosphorylation (Tyr632) of IRS-1 was increased while serine phosphorylation (Ser636) in the IRS-1 was decreased. This may be due to an increase in IR protein expression witnessed in the current study. Thus an aqueous leaf extract of *A. indica* has rich bioactive constituents which may play a vital role in the management of type-2 diabetes mellitus (Satyanarayana, Sravanthi, Shaker, & Ponnulakshmi, 2015).

17.4 Antimicrobial properties of *A. indica* (neem tree)

Some of the bioactive compounds obtained from the plant are actually not produced by the plant itself but by some microorganisms (entophytes). Fungi, bacteria, mycoplasma, and actinomycetes living inside plant tissues are responsible for the production of such compounds. Bioactive compounds belonging to the various classes of compounds are produced in order to protect the host plant from biotic and abiotic stress states. It is observed that the same plant grows in different habitats; this may lead to different types of endophytic microorganisms. *A. indica* was found to contain a number of bioactive compounds with antimicrobial activity which may be considered for the development of new drugs, as shown in Table 17.2 (Chatterjee, Ghosh, & Mandal, 2019; Kusari, Verma, Lamshoeft, & Spiteller, 2012).

17.4.1 Antimicrobial study by Siddiqui and coworkers

In a study, Siddiqui and coworkers investigated mahmoodin (Fig. 17.7) and its mother fraction (SF) by using 10 mg of each for antimicrobial potential evaluation against eight Gram-negative organisms and nine Gram-positive bacteria. Mahmoodin (**16**) exhibited promising inhibitory effects (zone of inhibition as mm in parentheses) against *Klebsiella pneumoniae* (15), *Shigella sonnei* (16), *Corynebarterium xerosis* (17), *Staphylococcus aureus* (17), *Bacillus cereus* (18), *Salmonella schotmuellwi* (20), *Barillus subtilis* (20), *Streptococcus faecalis* (22), and *Streptocxcus pyogena* (28). In contrast, SF showed weak effective zone against many of these strains (Siddiqui, Faizi, & Siddiqui, 1992).

17.4.2 Antimicrobial study by Siddiqui and coworkers

Over the years, neem has been studied for its chemical constituents responsible for insect-repellant and insecticidal properties. Neem tree has shown a marked effect on a wide range of pests, including insects, nematodes, fungi, bacteria, and even a few viruses.

In a study, Siddiqui and coworkers investigated 16 triterpenoids obtained from neem plant against *Anopheles stephensi* Liston to identify the active components liable for the pesticidal activity. *A. stephensi* Liston (malarial parasite−carrier mosquito) used in this study is the second most common anopheline found in this region. The pesticidal activity considered as lethal concentrations (LC_{50}) of these constituents was determined against fourth-instar larvae of *A. stephensi*. Among all the tested samples, azadiradione (**17**), nimbocinol (**18**), 17β-hydroxynimbocinol (**19**), and azadirone (**20**) (Fig. 17.8) exhibited significant pesticidal activities with LC_{50} values of 15, 30, 15, and 10 ppm, respectively. Structure−activity relationship analysis revealed that ring A with 1-en-3-one system, an acetoxy moiety substitution at C-7, and a furan ring at C-17 has considerably contributed to the toxicity of neem compounds. Similarly, open-chain α-β-unsaturation with a carbonyl group or double bond in an open chain increased the activity of the compound. Moreover, deacetylation at C-7 showed a decrease in the toxicity (Siddiqui, Ali, Rasheed, & Kardar, 2003a).

17.4.3 Antimicrobial study by Siddiqui and coworkers

In another study, Siddiqui and coworkers isolated an unknown tetranortriterpenoid called meliatetraolenone (**21**), and a previously known compound, odoratone (**22**) as shown in Fig. 17.9. Both compounds were isolated from the methanolic extract of *A. indica* fresh leaves and evaluated against fourth-instar larvae of *A. stephensi*. Meliatetraolenone showed an LC_{50} value of 16 ppm while odoratone exhibited an LC_{50} value of 154 ppm (Siddiqui et al., 2003b).

17.4.4 Antimicrobial study by Chianese and coworkers

A. indica has been used traditionally against malaria since antiquity. The limonoids isolated from the neem plant have shown wide spectrum of bioactive properties, such as antibacterial, insecticidal, antitumor, and antiviral properties. In a

TABLE 17.2 Anticancer, antidiabetic, and antimicrobial compounds from *Azadirachta indica*.

Name of compound	Bioactivity	Reference
Nimbolide (**1**)	Anticancer	Kigodi et al. (1989)
28-Deoxonimbolide (**2**)	Anticancer	Kigodi et al. (1989)
7-Benzoylnimbocinol (**3**)	Anticancer	Kikuchi et al. (2011)
Epoxyazadiradione (**4**)	Anticancer	Kikuchi et al. (2011)
7-Deacetyl-7-benzoyl epoxyazadiradione (**5**)	Anticancer	Kikuchi et al. (2011)
Gedunin (**6**)	Anticancer	Kikuchi et al. (2011)
7-Deacetyl-7-benzoylgedunin (**7**)	Anticancer	Kikuchi et al. (2011)
28-Deoxonimbolide (**8**)	Anticancer	Kikuchi et al. (2011)
Ohchinin acetate (**9**)	Anticancer	Kikuchi et al. (2011)
Deacetilsalannin (**10**)	Anticancer	Gualtieri et al. (2014)
1,3-Diacetylvilasinin (**11**)	Anticancer	Gualtieri et al. (2014)
Azadiradione (**12**)	Antidiabetic	Ponnusamy et al. (2015)
Epoxyazadiradione (**13**)	Antidiabetic	Ponnusamy et al. (2015)
Gedunin (**14**)	Antidiabetic	Ponnusamy et al. (2015)
Meliacinolin (**15**)	Antidiabetic	Perez-Gutierrez and Damian-Guzman (2012)
Mahmoodin (**16**)	Antimicrobial	Siddiqui et al. (1992)
Azadiradione (**17**)	Antimicrobial	Siddiqui et al. (2003a)
Nimbocinol (**18**)	Antimicrobial	Siddiqui et al. (2003a)
17β-Hydroxynimbocinol (**19**)	Antimicrobial	Siddiqui et al. (2003a)
Azadirone (**20**)	Antimicrobial	Siddiqui et al. (2003a)
Meliatetraolenone (**21**)	Antimicrobial	Siddiqui et al. (2003b)
Odoratone (**22**)	Antimicrobial	Siddiqui et al. (2003b)
Azadirone (**23**)	Antimicrobial	Chianese et al. (2010)
Azadiradione (**24**)	Antimicrobial	Chianese et al. (2010)
Epoxyazadiradione (**25**)	Antimicrobial	Chianese et al. (2010)
Gedunin (**26**)	Antimicrobial	Chianese et al. (2010)
Deacetylgedunin (**27**)	Antimicrobial	Chianese et al. (2010)
Desmethyllimocin B (**28**)	Antimicrobial	Chianese et al. (2010)
Protoxylocarpin G (**29**)	Antimicrobial	Chianese et al. (2010)
Spicatin (**30**)	Antimicrobial	Chianese et al. (2010)
Neemfruitin A (**31**)	Antimicrobial	Chianese et al. (2010)
Neemfruitin B (**32**)	Antimicrobial	Chianese et al. (2010)

study by Chianese and coworkers, 10 limonoids (compounds **23**–**32**) were isolated from the fruits of neem and their antiplasmodial activities were evaluated, as shown in Fig. 17.10.

Furthermore, multidrug-resistant *Plasmodium falciparum* strains such as the CQ-sensitive (D10) and the CQ-resistant (W2) strains were employed and the structure–activity relationship was established in this limonoid class.

Azadirone (**23**), gedunin (**26**), and neemfruitin A (**31**) tested against the CQ-sensitive (D10) and CQ-resistant (W2) strains showed significant inhibition with IC_{50} values of 1.63 ± 0.23, 1.21 ± 0.30, 1.66 ± 0.37 μM and 1.31 ± 0.42,

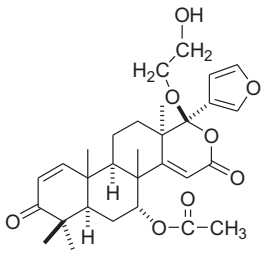

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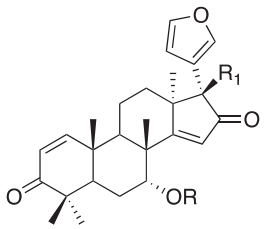
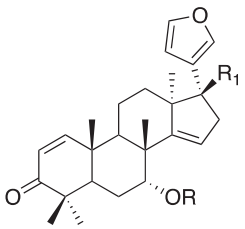
FIGURE 17.7 Antimicrobial compound identified by Siddiqui and coworkers.

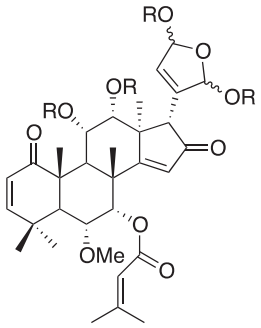


R = Ac, R_1 = H — Azadiradione **17**
R = H, R_1 = H — Nimbocinol **18**
R = Ac, R_1 = OH — 17β-Hydroxynimbocinol **19**

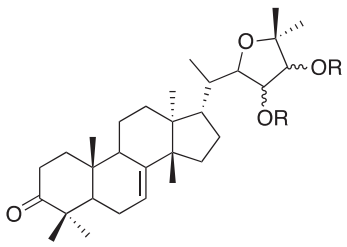


R = Ac, R_1 = H — Azadirone **20**

FIGURE 17.8 Antimicrobial compounds identified by Siddiqui and coworkers.



R = H
R = Ac
21



R = H
R = Ac
22

FIGURE 17.9 Antimicrobial compounds identified by Siddiqui and coworkers.

2.82 ± 0.70, and 1.74 ± 0.25 μM, respectively. While other tested compounds showed IC_{50} values ranging from 3.30 ± 0.75 to 9.49 ± 1.08 μM against D10 and 2.16 ± 0.73 to 9.98 ± 2.16 μM against W2 resistant strains, as shown in Table 17.3. The chloroquine was used as a reference with IC_{50} values of 0.03 ± 0.01 and 0.34 ± 0.09 μM, respectively. The variation in activities may be ascribed to the variations in the side chain, to the second conjugated carbonyl group at C-16, and to the presence of the furan ring (Chianese et al., 2010).

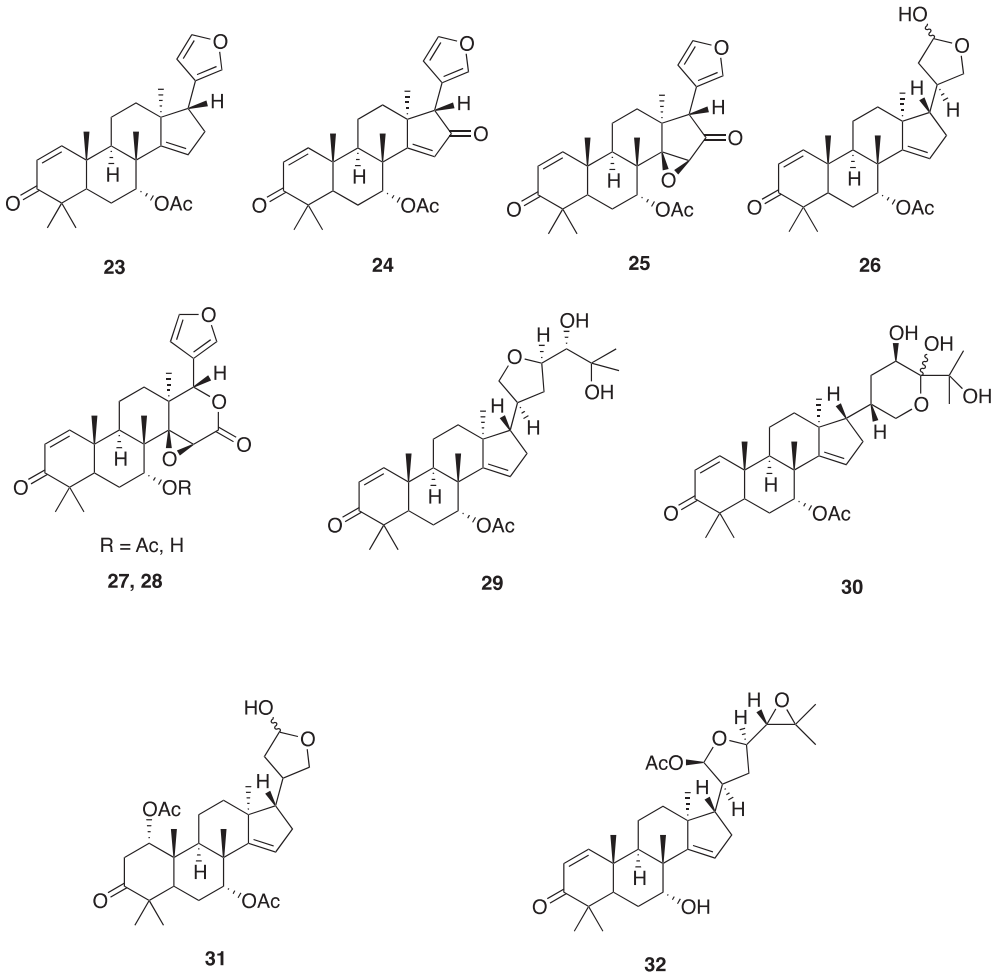


FIGURE 17.10 Antimicrobial compounds identified by Chianese and coworkers.

TABLE 17.3 Antimalarial activity (in vitro) of the triterpenoids (μM) from the fruits of *A. indica* tested against D10 (chloroquine sensitive, CQ-S) and W2 (chloroquine resistant, CQ-R) strains of *P. falciparum*.

Compounds	CQ- sensitive (D10)	CQ-resistant (W2)
Azadirone (**23**)	1.63 ± 0.23	1.21 ± 0.30
Azadiradione (**24**)	5.96 ± 0.76	3.40 ± 0.82
Epoxyazadiradione (**25**)	3.30 ± 0.75	2.16 ± 0.73
Gedunin (**26**)	1.66 ± 0.37	1.31 ± 0.42
Deacetylgedunin (**27**)	5.14 ± 1.23	3.29 ± 0.59
Desmethyllimocin B (**28**)	4.80 ± 0.53	2.59 ± 0.70
Protoxylocarpin G (**29**)	5.00 ± 0.66	2.40 ± 0.90
Spicatin (**30**)	5.40 ± 0.75	2.74 ± 0.79
Neemfruitin A (**31**)	2.82 ± 0.70	1.74 ± 0.25
Neemfruitin B (**32**)	9.49 ± 1.08	9.98 ± 2.16

17.5 Conclusions

A. indica (neem) is a versatile plant which is widely distributed all over the world. It is an important source of several types of compounds with diverse chemical structures and unique medicinal applications. Each part of the plant has medicinal properties that can be used to treat a wide spectrum of diseases. Crude extracts from different parts of the neem plant have been used traditionally to treat a variety of human health problems since ancient times. Therefore broad investigation of its bioactivity, mechanism of action, toxicity, pharmacotherapeutics, adequate standardization, and clinical trials are highly desirable to validate the known bioactivities of this "wonder tree" and to develop modern drugs. In fact, during the last few decades, huge efforts have been made to explore the chemical constituents of the neem plant and their therapeutic applications against cancer, diabetes mellitus, and certain microbes which lay the foundations for the adoption of modern approaches for its optimal domestic, industrial, and therapeutic utilization.

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Chapter 18

Advancements in plant transgenomics approach for the biopharmaceutics and vaccines production

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18.1 Introduction

With the production of plant made pharmaceuticals (PMPs) from transgenic plants a whole new industry of biopharmaceuticals is on the brink of origination (Yano & Takekoshi, 2004). The therapeutic agents produced via utilization of biotechnological tools can create a hallmark in the commercialization of products as they overcome the drawbacks that current microbial and animal expression systems face. Genetic engineering of plants has helped in the improvement of crops and agriculture in the last few decades and now the concept of edible vaccines and drugs from transgenic plants holds great interest to the researchers and biopharmaceutical companies (Ahmad et al., 2012). Today, plant-based expression systems are also being developed to act as bioreactors for vaccine antigens production; vaccines developed this way have already reached phase II and III of clinical trials. An example is the production of a vaccine against pneumonic plaque using plant-derived plaque vaccine (Alvarez & Cardineau, 2010) which requires a chloroplast-based expression system (Arlen et al., 2008).

The idea of utilizing crop plants as therapeutic food and fiber has evolved with the development of biotechnology tools. Transgenic plants for generating drugs, vaccines, dietary supplements, diagnostic tools, diet formulating agents, etc. are predicted soon to complement the conventional drugs available in the market (Raskin et al., 2002). This review will focus upon the advancements that took place almost over last two decades in molecular farming strategies for drugs and vaccine development. Particular emphasize will be laid on the different strategies and biotechnological tools that are being employed for the development of transgenic plants and novel expression systems designed for the production of plant-derived pharmaceuticals (PDPs) and vaccine antigens.

The concept of edible vaccines is now gaining great interest among researchers as they hold a huge potential for herd immunization, particularly in developing countries where transgenic crops with immunogenic antigens against various diseases can be used as a source of food. Such vaccines are not only cheap to produce but they also hold greater commercialization potential than other vaccine production sources (Ahmad et al., 2012).

This review covers two aspects of plant transgenomics for therapeutic drugs development, firstly "transgenic plants in development of biopharmaceutical agents" and secondly, "the vaccines derived from transgenic plants" to get an outcome that is "the commercialization and development of plant-derived pharmaceuticals and vaccines." Hence, this chapter focuses on the use of plant transgenomics techniques that can be employed in biopharmaceutical and vaccine industries. To the best of our knowledge, no review articles have been available on plant transgenomics on this aspect of its implementation in biopharmaceutical and vaccine sciences.

The literature review involved an extensive search for the synthesis of vaccines and pharmaceutics using plants as an expression system through four dimensions that were set in the theoretical framework. In the first search broad terms such as plant and drugs development and plants and vaccine production were studied and included, while in the second

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phase of searching medicinal plants were eliminated from the search and vaccines and pharmaceuticals being derived from plants as well as commercialized were primarily focused upon.

18.2 Transgenic plants in biopharmaceuticals

With the current advancements in biotechnological tools it has now become possible to develop genetically modified (GM) plants as a bioreactor system acting as a source of large-scale protein production for therapeutic purposes (Goldstein & Thomas, 2004). This large-scale production of industrial or biopharmaceutical products via the use of GM plants is known as a process of "plant molecular farming" (PMF) (Fig. 18.1) (Wang & Ma, 2011). The GM plants used to produce these "high-value molecules" are included in third-generation GM crop plants which include antibodies, vaccines, immunomodulatory drugs, etc., or those plants which are GM to act as nutraceuticals, such as the famous "golden rice." These PMPs are acting as a hallmark of drug development (Hoffmann-Sommergruber, 2012).

In order to meet the increasing demands for biopharmaceutical products, plant-derived pharmaceuticals (PDPs) can be synthesized in a cost-effective manner. PDPs are easier to produce in mass-scale processes, with greater safety and efficacy than their animal-derived counterparts (Kaloudas & Penchovsky, 2018; Spök, Twyman, Fischer, Ma, & Sparrow, 2008). An example is that of transgenic tobacco-derived secretory antibody that has already been found to be effective against bacterial pathogens for topical application inside the mouth in clinical trials (Warzecha & Mason, 2003). More examples include plant-based antifibrotic agents, developed to reduce oxidative stress in liver fibroid cells (Gebhardt, 2002) and recombinant allergens such as house dust mite allergen produced from transgenic tobacco and Japanese cedar pollen allergens produced from transgenic rice seeds (Schmidt et al., 2008).

Proteins derived from GM plants are fully functional with an identity equivalent to their mammalian derivatives. Hence, bacterial and other microorganisms-derived therapeutic agents which lack the posttranslational modifications can be easily generated via PMF procedures. Mammalian systems, which have the occasional hazard of pathological contamination, for example, viral antigen expression, can be replaced by PMPs since these products are free of mammalian oncogenic DNA sequences induced in mammalian-based systems through transgene strategies and endotoxins commonly secreted in microbial-based systems (Goldstein & Thomas, 2004). Hence safety, efficacy, cost-effectiveness, and mass-scale production of therapeutic agents are key advantages in developing a plant biopharmaceutical bioreactor system.

There are several ways through which modulation of a gene expression system in a plant model can be carried out. Researchers have been trying to develop different plant models as an efficient expression system for biologically active proteins. Extensive experimentation and research has been carried out on different plant hosts and different sites for transgene insertion have been studied for efficient results (Goldstein & Thomas, 2004; Yano & Takekoshi, 2004).

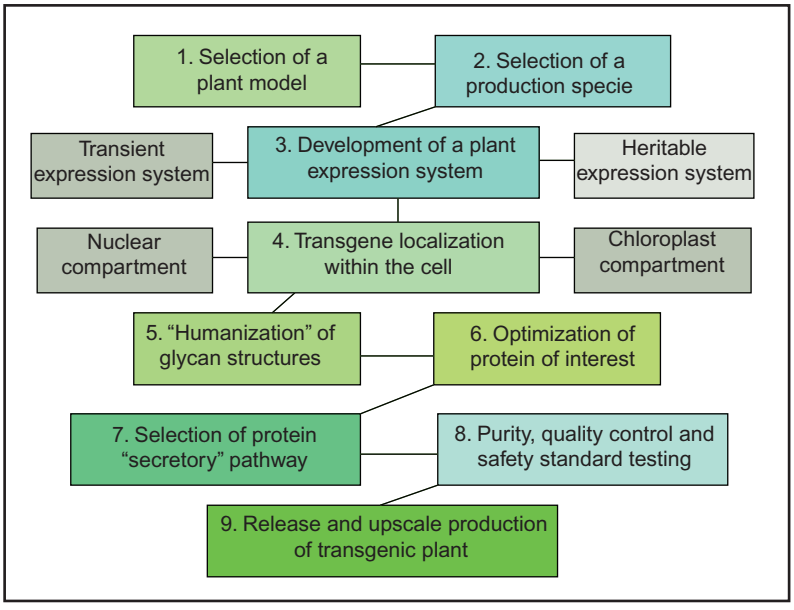

FIGURE 18.1 The chart illustrates various steps in molecular farming approach that can be employed to develop a plant expression system for plant made protein and pharmaceutics development.

18.2.1 Selection of a plant model and production species

Plant model selection is based on several factors, such as an ease of development, techniques available for the genetic transformation, high-scale production of therapeutic agents, organ harvesting, and processing of PMPs. An example is that of tobacco plant which is the plant of choice for green matter synthesis as it has a high biomass production rate (Goldstein & Thomas, 2004). For a high amount of recombinant protein extraction the seeds-based PMP production is preferred which includes the choice of cereals such as maize (Moeller et al., 2010), legumes (Peters & Stoger, 2011), barley, wheat, and rice (Takaiwa, Takagi, Hirose, & Wakasa, 2007). Further, crop plants, such as soybean (Vianna, Cunha, Murad, & Rech, 2011) and peas (Mikschofsky & Broer, 2012), can be also be used for antibodies, hormones, and vaccines production (Giddings, Allison, Brooks, & Carter, 2000).

According to Twyman, Stoger, Schillberg, Christou, and Fischer (2003) there are four plant species that are being targeted at present for the plant transgene strategy.

18.2.1.1 Leaf part of crop

Tobacco plant was one of the earliest plant species that was exploited to produce therapeutic proteins via molecular farming strategies. At present, it is being developed at the commercial level by various companies to produce various pharmaceutical proteins, for example, sIgA"caroRx" by Planet Biotechnology for remediating tooth decay, TGF-beta protein for treatment of ovarian cancer by Chlorogen & partner, β-glucosidase protein production by TransPharma srl and Plantechno srl for treatment of Gaucher's disease, and the development of antiidiotype IgG antibodies by Bayer Innovation (Hoffmann-Sommergruber, 2012; Sharma & Shahzad, 2013). Tobacco is a plant of choice for these products owing to the following properties: the high yield potential, an ease of cultivation, efficient gene transfer, high biomass and seed production. Tobacco is not a feed crop, hence the chances of contamination of the food chain by any transgenic plant product is highly unlikely. The only disadvantage of the use of tobacco is perhaps the synthesis of phenol-based compounds, along with recombinant proteins which can be eliminated by downstream processing (Gruber & Theisen, 2000).

Other leafy plants that had been explored to produce PMPs include alfalfa and soybean. They both provide a preferential advantage of being nitrogen-fixing plants, hence lowering the need for chemical fertilizers during cultivation. In addition, the high biomass yield per hectare allows these legumes to be of particular importance for recombinant antibodies development. Soybean is also used for lactoferrin production for use in infant formula by Plantechno srl, while Medicago co. is using alfalfa as an expression system for the production of several recombinant proteins (Hefferon, 2012).

18.2.1.2 Seeds from crop plants

The seeds system provides several unique properties to the plant expression system, which include a high amount of recombinant protein can be expressed within seeds with long-term expression and storage (Boothe et al., 2010). Cereal and legume crops are being developed at present for PMPs production. Cereals (rice, wheat, and maize) and legumes (pea and soybean) have the ability to accumulate and preserve recombinant proteins which provides easy production of temperature-resistant products. Hence problems associated with other plant species, such as loss of activity and need for rapid processing can be rapidly overcome. Exposure to insects, animals, and other nontarget organisms can also be avoided using recombinant seed-based systems. Maize plant seeds were used for the first time to undergo molecular farming and at present they are being used at the commercial level for insulin and trypsin production by Podigene. Recombinant avidin and B-glucuronidase are also being produced at commercial level via the use of maize. Rice is also another important target for protein product manufacture, particularly due to its high productivity rate and ease of upscaling in commercial settings; an example is that of Apo-A1 (Milano) production for cardiovascular disease treatment by Plantechno srl (Sharma & Shahzad, 2013).

Using commercial crops such as flax and cotton and oil crops such as oilseed rape reduces the processing cost for recombinant protein production. Commercial crops are now being used at a large scale for drug development, for example, flax is being used by Agragen to produce human serum albumin (Hoffmann-Sommergruber, 2012).

18.2.1.3 Fruit and vegetable crops

With the development of the expression system inside a fruit and vegetable crop, a whole new biopharmaceutical production era will arise for the benefit of humans. The transgenic plants and vegetables could be consumed as a whole, partially cooked, and/or processed food and can function as a recombinant subunit vaccine vector and antigen delivery

system, antibodies producer, and as a source of nutraceuticals. The introduction of vitamin A in "golden rice" has already been tested. Biofortification strategies can be employed to address malnutrition issues in developing countries. For eradication of vitamin A deficiency in developing countries, GR-1 had been developed by researches containing three new genes, two from daffodil (*Narcissus pseudonarcissus*) and one from a bacterium (*Erwinia uredovora*) (Lemaux, 2008). It is an efficient demonstration of how a transgenic plant can act on a large scale as a biomedical substance, when the crop is directly used as a food source. Similarly, the concept of using transgenic potato tubers (Rukavtsova, Chebotareva, Rudenko, & Buryanov, 2011) as a vaccine antigen delivery vector has already met clinical trials on three different occasions. Similarly, tomatoes and bananas are also potential vaccine delivery vehicles. Protalax co. is currently using carrot cells to produce several products, such as glucocerebrosidase, acetylcholinestrase, and alpha-galactosidase and antitumor necrosis factor.

18.2.2 Expression system

Three gene transfer systems are being employed at present to develop transgenic plants of interest.

18.2.2.1 Transient expression system

There are three methods that can be employed to carry out transient expression (TE) of a gene inside a transgenic plant: (1) ballistic gene gun method; (2) agroinfiltration using *Agrobacterium tumefaciens*; and (3) viral delivery method. Fig. 18.2 depicts the methodology adopted for transgenic plants production and process from the selection of plant species to large-scale commercial production of plants for biopharmaceutics and vaccine development.

In the TE system, the genetic material does not get incorporated inside the plant chromosome and fresh production of transformed cells is required to allow for the protein production (Komarova et al., 2010). Hence, TE systems can be useful, when verification of expression constructs is required and when rapid production of protein is the target, such as for research purposes, functional analysis, and drug designs, before proceeding to its commercial and large-scale protein

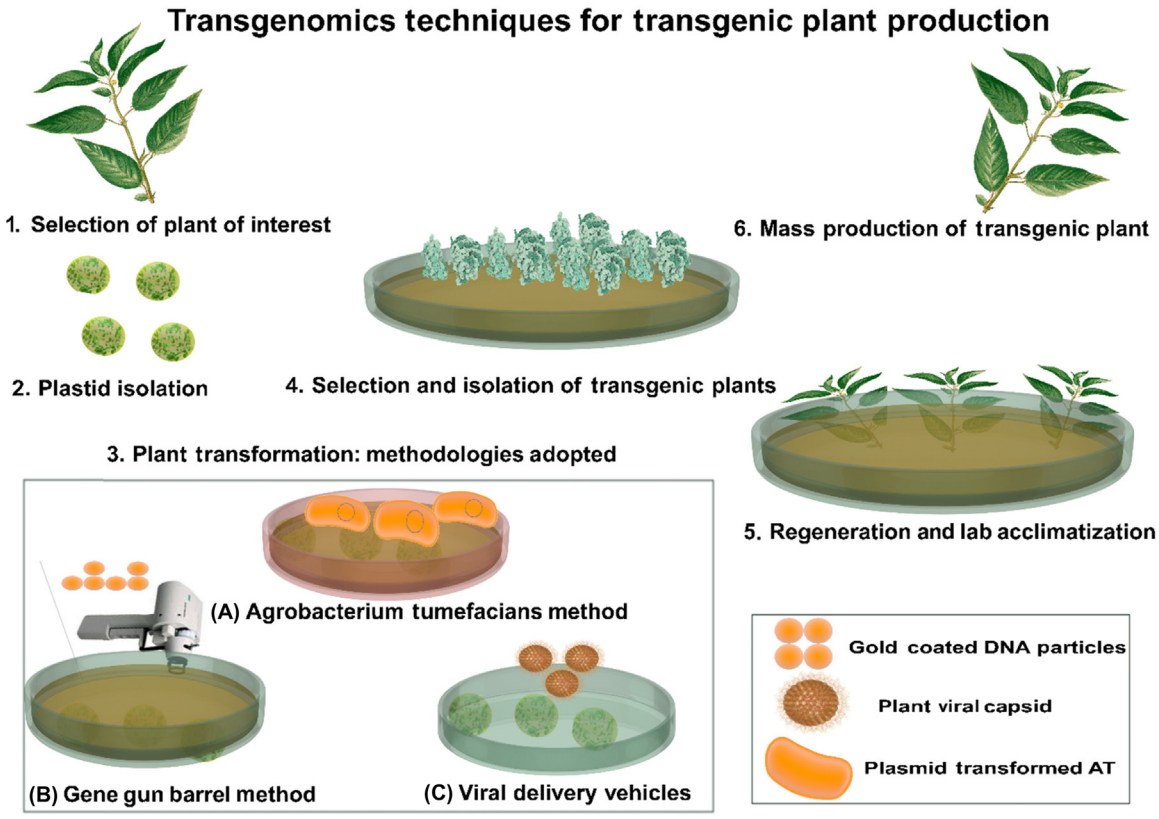

FIGURE 18.2 Transgenomics techniques involved in transgenic plants development and commercialization of biopharmaceutics/vaccine production. There are three methodologies adopted for plant transformation: (A) *Agrobacterium tumefaciens* mediated method; (B) Biolistic shotgun method; (C) Viral delivery system.

production. However, the TE system involving the agroinfiltration method has been found to be useful in tobacco plants for high-scale protein production (Goldstein & Thomas, 2004; Twyman et al., 2003).

In the viral−host system strategy, the recombinant viral particles are used to infect a plant host. As the virus replicates inside host plant cells, transgene expression occurs along with the viral gene expression (Giddings et al., 2000). At present, plant−vector systems are being developed to produce monoclonal antibodies, carry the epitopes for subunit vaccines (Hefferon, 2012), and to produce dietary proteins with high efficacy and safety (Yusibov, Streatfield, & Kushnir, 2011). This system is particularly attractive due to the shorter time scale required to express a substantial level of the gene of interest, which can be further scaled up when the number of host plants is increased, while maintaining the rapid production and ease of purification of the gene of interest (Hefferon, 2012). An example of such a system is the use of tobacco mosaic virus to infect tobacco plants and cowpea mosaic virus to infect cowpea plants. Other viruses include potato virus X, alfalfa mosaic virus and cucumber mosaic virus (Yusibov et al., 2011).

However, the transient expression system has one potential drawback: whole transgenic plant leaves provide varied levels of protein expression, hence it could result in uncertainty in product manufacturing and can increase the regulatory burden, as well as purification and production cost. Optimization of protein levels has to be carried out before transient transgenic plants could be utilized for the commercial development of recombinant proteins (Buyel & Fischer, 2013).

18.2.2.2 Heritable expression system

Primary plant chromosomes can be modified for heritable expression of a protein and hence a stream of transgenic plant species could be generated for PMPs production at a commercial level. *A. tumefaciens* is commonly used for gene insertion by making appropriate modifications inside a pathogen genome (Goldstein & Thomas, 2004). *Nicotiana tabacum* and *Arabidopsis thaliana* have been extensively established as plant model systems for transgenic plants synthesis (Giddings et al., 2000). For dicots, such as tobacco and peas, and some of the monocots, such as rice, *Agrobacterium*-mediated conversion remains the method of choice for transgene construct. The agrobacterial transformation is based on regeneration of whole plants from cells transformed with vectors based on T-DNA of agrobacteria (Matveeva & Sokornova, 2017).

The ballistic or metallic shotgun method is also being used to produce transgenic plants, where genetic material coated onto small metallic pellets is introduced directly inside plant cells and gets incorporated into the genetic material of plant (Giddings et al., 2000). Monocots, such as wheat and corn, are usually converted using this method. However, screening for efficient transfer and transgene plant construct and protein of interest expression has to be carried out before the plant can be used for PMPs production. Stable transformation of a plant is a time-consuming process and a plant variety generated this way can be available in almost 3−9 months for testing the protein of interest, hence for research purposes a prerequisite TE system is preferred before moving toward the heritable transgene plant construct (Wang & Ma, 2011).

The choice for a plant expression system depends on several factors, such as the efficiency of transgenic plant synthesis, the cost of development, the quantity of recombinant protein required, safety issues associated with release of such crops, public acceptance, and production requirement (Goldstein & Thomas, 2004; Twyman et al., 2003). Fig. 18.2 explains three gene transfer methods currently being employed to create transgenic plants.

Fig. 18.2 explains in six steps how three different gene transfer methods can be used in vitro to create two different types of expression systems in transgenic plants which can then be optimized and upscaled for biopharmaceutics and vaccine production.

18.2.3 Transgene location

For heritable expression of a transgenic plant construct it is crucial to decide upon the final location of a transgene. Chloroplast and nuclear compartments can be targeted for a transgene insertion that can then be passed on to future generations. At present, both sites are being used to produce biopharmaceuticals of interests (Hefferon, 2013). The introduction of a transgene inside chloroplast DNA is a considerably novel idea that has been implicated to have several advantages over nucleus DNA conversion. First, each cell contains hundreds of these compact, energy packets, hence they have an ability to produce functional copies of transgene, increased manifold to the conventional nucleus copies produced per cell in a plant (Scotti & Cardi, 2012). Tobacco chloroplasts, for example, can produce human somatotropin at protein levels over one hundred times higher than their nuclear transgenic counterparts (Goldstein & Thomas, 2004). Furthermore, plant transgenes have an ability to produce uniform gene products, as epigenetic factors do not

interfere in plasmid genes. In addition, multiple genes can be expressed at the same time from a single construct with a substantial environment-friendly protein source, as the plastids are maternally inherited so the chances of horizontal gene transfer across the species via pollen are negligible (Daniell, 2006; Scotti & Cardi, 2012). Hence, plastid transformation technology is being developed as an alternate source for biopharmaceutical production using the transgenic plant construct. For the nuclear insertion of a transgene, the problems associated due to gene silencing have to be addressed and protein expression levels optimized before the commercialization of nucleus-localized transgenic plants (Buyel & Fischer, 2013).

18.2.4 Humanization of glycan structures in products

Biopharmaceutical products present maximum efficacy in glycoprotein form when administered in humans for a therapeutic purpose. As the *N*-glycosylation Golgi apparatus of the mammalian cell varies from that present in plants then in order to develop a plant transgene system for mammalian biopharmaceutics synthesis, several changes have to be introduced to allow the conversion of plant *N*-glycoforms to the mammalian counterparts (Karg & Kallio, 2009). As in the mammalian system, proper folding as well as disulfide bond formation takes place inside a chloroplast, or in the case of nuclear gene, inside endoplasmic reticulum, when using a transgenic plant construct. Lipid modifications also take place inside a transgenic plant particularly, when using a chloroplast transformation system (Daniell, Singh, Mason, & Streatfield, 2009). Strategies are being developed that can be used to "humanize" the glycan structures from recombinant glycoprotein constructs from transgenic proteins. It includes (1) the in vitro use of purified human b(1,4)-galactosyltransferase and sialyltransferase for modification of recombinant proteins that are derived from transgenic plants, and (2) when constructing recombinant antibodies with galactose/extended glycans in transgenic plants, human genes containing coding for b(1,4)-galactosyltransferase can be expressed along with recombinant protein. However, it had also been noticed in few cases that the use of plant glycans in clinical settings confer improved performance of the drug and may actually provide unique, differential properties to drugs that may be absent in mammalian-based counterparts (Melnik & Stoger, 2013). Therefore the glycosylation reaction of a recombinant protein has to be optimized according to the required therapeutic effect in humans.

18.2.5 Optimization and secretion of protein of interest

Expression levels of a protein can be scaled up by controlling the innate plant system involved in protein sorting and targeting. A significant increase in recombinant antibody production had been noted when a protein was tagged for the secretory pathway instead of cytosolic. It has also been observed that targeting a protein for localization into apoplast space along with high protein retention time in endoplasmic reticulum increases the protein production rate 10−100-folds.

Many systems have been developed to monitor the synthesis and control the optimization of recombinant proteins; green florescence protein (GFP) tags had been used in one study to monitor stable transformation of plant. GFP-tagged recombinant proteins can also be optimized by measuring the florescence intensity, which corresponds to the level of protein production (Li & Chye, 2009). Another florescence protein known as Ds Red, providing high detection sensitivity, is also being readily used as a gene-marker for the transformation, optimization, and selection of transgenic maize plants. Later on the Ds Red tagged recombinant protein can also be used to monitor successful transformation of germplasm during breeding for future transfer of traits (Rademacher, Arcalis, & Stoger, 2009).

At present, three secretory sites are being developed in plant cells: ER, chloroplast, and apoplast (Fischer & Emans, 2000). The choice of secretory pathway depends upon the nature of the recombinant protein being produced and the transgene insertion site, however, high protein yield is the prime target for the selection of any particular route. Two important factors govern the overall yield of recombinant protein: one, as discussed above, is dependent upon the expression system and route of protein secretion, while the second is dependent upon the protein product itself (Tackaberry et al., 2003).

18.2.6 Purity, quality control, and safety standard tested

Two hurdles have to be countered before PDPs can be purified from their transgenic plant hosts: (1) each plant system provides a different array of impurities which include nucleic acid debris, phytic acids, phenolic compounds, proteins, and fatty acids that have to be removed according to the custom demand of each plant system; and (2) target proteins accumulate in low amounts in a medium after initial purification which has to be scaled up for commercial settings

(Menkhaus & Roseland, 2008). Many types of purification techniques can be employed, including selective adsorption and elution when separating recombinant proteins from the transgenic plants.

In order to extract proteins from seeds, an oleosin-fusion platform as developed by SemBioSys Genetics Co. can be employed, since fusion recombinant proteins are embedded in the cellular membrane after production, which can then be eluted though appropriate use of buffers or detergents to isolate the protein of interest. Similarly, Phytomedics Co. has developed a system for collection of human-secreted alkaline phosphatase which can be employed for the collection of recombinant protein exudates from vegetative parts of plants such as roots and leaves (Twyman et al., 2003).

Hence, purification of protein from transgenic plants in a cost-effective way is a crucial point in a PMPs construct that has to be addressed before the commercialization of pharmaceutically active transgenic plants.

18.2.7 Release and agricultural-scale cultivation of transgenic plants

At present, the commercialization of plant-based biopharmaceuticals is still in its infancy as several issues associated with the upscaling of protein products have to be addressed before the commercial release of transgenic plants. Vaccines derived from plants have been developed over the last two decades and only a few of them have reached clinical trials for human utilization (Daniell et al., 2009). However, much effort is being made at present to overcome the technological problems associated with the molecular farming methods currently employed to develop plant transgenic systems. The release of such crop plants in agriculture settings demands other issues to be addressed, which include biosafety hazards, ethical considerations, risk assessment, environmental impact of crop release, and public acceptance for such edible biopharmaceuticals (Twyman et al., 2003).

18.3 Transgenic plants in vaccine development

Prevention and control of infectious diseases might become possible through global vaccination and herd immunization programs. At present, around 20% of the child population around the world is deprived of vaccination programs, which results in two million deaths every year (Lal, Ramachandran, Goyal, & Sharma, 2007). The main issues associated with the lack of immunization programs, particularly in developing countries, are restraints on vaccine manufacture, distribution, and administration (Lal et al., 2007; Thanavala, Huang, & Mason, 2006). Vaccination is either nonexistent in developing countries or unreliable if present and costly for some of the infectious diseases. So, there is a quest for new methodologies to make vaccines accessible and speed up immunization programs; these methods should be reliable to use, accessible to the general population, safe to handle, easy to administer, and they should be economical and cost effective (Saroja, Lakshmi, & Bhaskaran, 2011; Silin, Lyubomska, Jirathitikal, & Bourinbaiar, 2007). The concept of using transgenic plants as a source of vaccines provides needle-free vaccine delivery in addition to the economical synthesis of therapeutic proteins (Rice, Ainley, & Shewen, 2005; Rybicki, 2010). The idea to use edible crops as vaccines has also emerged as a means for herd immunization against epidemic diseases in developing countries (Hefferon, 2010). Plant-based vaccines could also provide preventive measures against cancer and autoimmune diseases development (Sala et al., 2003). These vaccines have several improvements when compared with conventional vaccine development strategies, for example, the low cost of production, ease of synthesis and isolation, efficacy in administration, and storage (Mei, Tao, Yuan-Gang, & Zhi-Gang, 2006). Currently two types of expression system are being employed for virus-like protein (VLP) productions in plants: stable and transient expression. VLPs produced in the plant systems as a vaccine are considered to elicit a potent and safe immune response compared to the other synthetic vaccine-based approaches (Marsian & Lomonossoff, 2016). However several issues associated with the efficacy of plant-based vaccines still need to be addressed, for example, immunological responses and allergic reactions while producing anticancer vaccines (Hooper, 2009). Tables 18.1–18.3 list some of the examples of vaccines that are being developed in different transgenic plant sources to combat infections and diseases worldwide.

18.3.1 Plants for vaccine expression

A large variety of plants have been tested for the expression of vaccine antigens, including fruits and tubers (tomato, potato), edible leafy crops (alfalfa, spinach), seeds (maize, rice), nonfood crops (tobacco), maize seeds, chloroplasts, and tobacco cell cultures. Transgenic rice plant has been used to produce recombinant *Helicobacter pylori* urease subunit B vaccine (Gu, Han, Liu, & Zhu, 2006) while a lettuce plant expression system has been used to produce synthetic cholera toxin B (CTB) subunit vaccine (Huy, Yang, & Kim, 2011). Vaccine-related products from tobacco and

TABLE 18.1 Highlights of the current review.

Transgenic plants	Transgenic plant's role in biopharmaceutics	Medicinal plants	Outcome of the included research
The role of transgenic plants as an expression system	Plant-derived pharmaceutics (PDPs) and transgenic plants for vaccines development[a]	Plants that have inherent medicinal properties were excluded from study	The research undertaken for commercialization and development of TP-derived biopharmaceuticals

[a]*Only plant-derived pharamceutics and their role as transgenic plants for vaccine development were included in the study.*

TABLE 18.2 Commercially available pharmaceutical products from plant expression system.

Crop plants modified for expression system	Pharmaceutical product synthesized	Treatment of disease	Clinical stage of product testing	References
Maize	Gastric lipase	Cystic fibrosis	Phase II	Aloulou and Carriere (2008)
Maize	Lactoferrin	Gastrointestinal disorders	Phase I	Samyn-Petit et al. (2001)
Tobacco	sIgA "CaroRx"	Prevention of tooth decay	Phase II	Gruber and Theisen (2000)
Maize	Trypsin	Wound care/ insulin manufacture	Available in Sigma catalogue '07	Woodard et al. (2003)
Rice	Lactoferrin	Infant formula enhancer	Available for sale	Nandi et al. (2002)
Lemna	Alpha interferon	Hepatitis "B" and "C" and cancer	Phase II	Cox, Dickey, and Gasdaska (2010) and De Leede et al. (2008)
Lemna	Recombinant plasmin	Fibrinolytic "Clot-buster"	Phase I	De Leede et al. (2008)
Arabidopsis	Human intrinsic factor	Vitamin B12 deficiency	Successful clinical trial and cGMP production certified	Fedosov et al. (2003)
Arabidopsis	DesB(30)-insulin	Diabetes	Preclinical	Nykiforuk et al. (2006)
Carrot cell	Glucocerebrosidase	Gaucher's disease	On sale	Shaaltiel et al. (2007)
Maize	Antibody	Cancer	Phase II	Hood, Woodard, and Horn (2002)
Safflower	Apolipoprotein AI	Cardiovascular	Phase I	Nykiforuk et al. (2011)
Rice	Apo-A1 (Milano)	Cardiovascular disease	Preclinical	Fogher, Reggi, and Perfanov (2017)
Soya	Lactoferrin	Infant formula enhancer	Preclinical	Arakawa, Chong, Slattery, and Langridge (1999)
Lettuce	Hepatitis B surface antigen	Hepatitis B	Phase I	Kapusta et al. (1999)
Lettuce	B subunit of Shiga toxin 2e	Edema	Preclinical	Matsui, Asao, Ki, Sawada, and Kato (2009)
Tobacco	Antiidiotype IGg Antibody	Non-Hodgkin's lymphoma	Phase I	Bendandi et al. (2010)

The table highlights some of the plant-based pharmaceutics developed at commercial level using transient plant-derived expression system.

TABLE 18.3 Plant systems tested for the expression of vaccine antigens and proteins.

Plant host	Vaccines developed	Treatment of disease	References
Maize	Lt-B vaccine	*Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT)-associated diarrhea	Chikwamba et al. (2002)
Tobacco	Vaccine	Non-Hodgkin's lymphoma	McCormick (2011)
Tobacco	Recombinant glycoprotein B (gB)	Human cytomegalo-virus	Tackaberry et al. (2003)
Potato	*E. coli* vaccine	Enterotoxigenic *E. coli*	Tacket (2009)
Tomato	TBI-HBS vaccine	HIV-1 and hepatitis B surface antigen	Salyaev, Rekoslavskaya, Shchelkunov, Stolbikov, and Hammond (2009)
Tomato	Recombinant Norwalk virus (rNV) capsid protein vaccine	Norwark virus-associated gastroenteritis	Zhang, Buehner, Hutson, Estes, and Mason (2006)
Tomato	Edible subunit malarial vaccine	Malaria	Pinzon-Charry (2007)
Rice	*Chlamydophila psittaci* vaccine	Avain chlamydiosis	Zhang et al. (2009)
Spinach	Vaccine	Rabies	Yusibov et al. (2002)
Tobacco	Rotavirus-like particle vaccine	Rotavirus associated gastroenteritis	Yang et al. (2011)
Potato tuber	Hepatitis B surface antigen	Hepatitis B	Thanavala and Lugade (2010)
Tobacco protoplast	HIV nef	HIV prophylactic	Marusic et al. (2007)
Nicotiana benthamiana	Recombinant Hemagglutinin A	Influenza subunit vaccine	Fujiuchi, Matsuda, Matoba, and Fujiwara (2017)

carrot cell suspensions have already been trialed for commercial-scale production and are expected to meet clinical trials soon (Franconi, Demurtas, & Massa, 2010). A new avenue had also been introduced by edible vaccines to treat atherosclerosis through the production of apolipoprotein B100 (ApoB100) and the choline esterase transferase protein (CETP) antigens inside transgenic plants; this altered method of administration might provide patient compliance and an environment-friendly approach in the synthesis of atherosclerosis vaccine (Salazar-González & Rosales-Mendoza, 2013).

18.3.2 Subunit vaccines

At present subunit vaccines are being developed in mammalian and bacterial cell cultures and research is being carried out for the development of a plant-based expression system. Hepatitis B subunit antigen (HbS Ag) expression inside the lettuce plant is an example of how a subunit vaccine could be developed using transgenic plants (Sala et al., 2003). In another study, HbS Ag has also been developed and its immunogenecity tested in a potato tuber (Rukavtsova et al., 2011).

Vaccines are also being developed to combat veterinary diseases, such as new castle virus disease and foot and mouth disease. Research has been carried out to develop a subunit vaccine against FMD virus in transgenic rice plant through an agrobacterium-mediated transformation system. Recombinant protein translated was then purified from transgenic rice plant and its immunogenecity was tested in a mice model; it was found to be effective in generating ample immune response against FMD virus (Kumar, Deepesh, Mahavir, & Archana, 2012; Wang et al., 2012). Similarly, subunit vaccines for fish can also be synthesized to maintain a safe aquaculture using transgenic plants in future (Clarke, Waheed, Lössl, Martinussen, & Daniell, 2013).

It was found that plant-derived antigens for subunit vaccines can massively enhance mucosal as well as serum antibody titer (Mason & Herbst-Kralovetz, 2011). Genetic fusion of recombinant proteins derived from recombinant plants can also allow for the production of chimeric proteins that can function as adjuvants as well as antigens for vaccine development (Soria-Guerra, Moreno-Fierros, & Rosales-Mendoza, 2011).

Plant-based influenza subunit vaccines are also emerging as a promising platform to express vaccine proteins. The scalability of production, efficiency in terms of time and labor costs, the lack of mammalian-associated pathogens, while retaining the eukaryotic machinery (including posttranslational protein modification) make plant-based expression systems an efficient SUVs developer system (Yusibov, Kushnir, & Streatfield, 2015).

18.3.3 Edible vaccines

Recently, there has been an accelerated need for needle-free vaccine delivery due to increased risk of bioterrorism, disease pandemics, and disease eradication campaigns (Giudice & Campbell, 2006). Owing to the drawbacks associated with conventional (live/attenuated/inactivated) vaccines, there is a need to find alternative methods to generate strong immunity to combat diseases (Gómez, Zoth, & Berinstein, 2009). It is well-known that oral administration elicits excellent immunological responses. Therefore oral delivery vaccine design utilizing genetically transformed plants is a potential substitute for conventional vaccines as animal pathogens and microbial toxins do not contaminate transgenic plants (Loza-Rubio & Rojas-Anaya, 2010). Edible vaccines also open up a great avenue for the synthesis of antiparasitic vaccines involved in gastrointestinal diseases through the induction of strong mucosal immunity and ease of development and target-site (GI-tract) administration (Wedrychowicz, 2000).

Selected genes are introduced for protein production during the transformation of transgenic plants. Edible vaccines do not code for genes for pathogenicity similar to subunit vaccines, so they cannot induce infection, especially in immunocompromised patients. These vaccines obviate the demand for trained medical staff, are genetically stable, do not require a purification step, and can be produced on a large scale (Lutwick, 2008). Such vaccines also eliminate the chances of infections spreading via needles and prions, such as in mad cow disease infection, because humans are not susceptible to plant viruses (Lavelle & O'Hagan, 2006).

Crop plants can act as vaccine antigen vectors for several diseases. In one recent study, it was hypothesized that tomatoes can act as an antigen delivery vehicle for malaria, which is not only a cost-effective method but an efficient way to help control malaria in developing countries (Chowdhury & Bagasra, 2007). On another occasion, experiments were conducted on corn and potato tubers to develop oral vaccines against diarrheal diseases (Tacket, 2007).

The site of entry for a variety of pathogens is the mucosal lining, which is also first line of defense against pathogens and a potential site for eliciting a first immunogenic response against any invading particle (Gómez, Zoth, Carrillo, Roux, & Berinstein, 2008). The purpose of an oral vaccine is to induce both mucosal and humoral immunity. Conventional oral vaccines after administration face problems regarding protein degradation in the stomach on exposure to acidic pH and do not efficiently elicit immune responses, but plants provide protection through cell walls and antigens are released in the intestine (Lal et al., 2007). Payer's patches present in intestine are mucosal effector sites, which are consistent upon lymphoid tissues. Ag upon entry interacts with M cells in Payer's patch inducing the expression of MHC-II on the surface and activating B-cells, which upon migration to the mucosal layer are differentiated into antibody-producing plasma cells. IgA immunoglobulin is secreted, which then blocks the interacting antigen, thereby generating immune response. The secretory IgA (SIgA) antibodies, highly abundant in the digestive and mucosal tract and the first choice for oral passive immunity, have been successfully produced in a plant-based expression system (Juarez, Virdi, Depicker, & Orzaez, 2016).

A practical example is the production of anti-TNF antibodies or glucocerebrosidase enzyme by the plant biotech company Protalix Bio therapeutics, which uses lyophilized carrot cell for the production of oral protein immunity as a therapeutic. Other examples include the approval by the US Food and Drug Administration (FDA) for three human clinical trials, where investigational new drug (IND) applications were filed and volunteers treated with raw potato tubers to establish antibody response specific to mucosal immunization (Arntzen, 2015). Other examples of lyophilized plant cells for oral immunization applications are also reviewed in detail by Chan and Daniell (2015).

18.3.4 Chloroplast-based vaccines

The plastid transformation process plays a crucial role in making this technology more technofeasible. It is the expression of high level of proteins in plastids and excision of expression markers (Lössl & Waheed, 2011). An increased protein level achievement is the major accomplishment for a practical -large-scale yield of oral vaccine. *tetC* antigen, when expressed in plastids from tobacco yielded approximately 25% of total soluble proteins (TSP) (Tregoning et al., 2003). Similarly, other antigens expressed themselves highly in plastids, accumulating from 26%−70% of TSP (Lössl & Waheed, 2011). The second major achievement is the excision of a transformation marker(s) which hinders the commercialization of such plants by raising environmental and health-related issues because these markers (e.g., *aadA* for

plastids) code for antibiotics (spectinomycin/streptomycin). Hence, the homologous recombination technique using direct repeats is applied for the marker excision by flanking the incorporated marker gene (Maliga, 2002).

The lack of posttranslational modifications and protein-folding makes the *Escherichia coli* system inefficient for recombinant protein expression. Plastids correctly fold protein antigens by forming −S−S− bonds that are crucial for a quaternary structure by maintaining their integrity and antigenicity (Cardi, Lenzi, & Maliga, 2010; Chebolu & Daniell, 2009). Viral and bacterial antigens are hence efficiently produced using chloroplasts.

Hepatitis C, cervical cancer, smallpox, and HIV are a few viral infectious diseases against which chloroplast-derived vaccines have been reported. The hepatitis C virus core protein has been expressed in plastids (Madesis et al., 2010), while a capsid protein (L1) of HPV-16 has been successfully expressed in tobacco chloroplast, which showed high immunogenicity in mice (Millán et al., 2008). This has a great impact on the treatment of the second most common cancer (cervical cancer) in females (Waheed, Gottschamel, Hassan, & Lössl, 2012). Recently, a synthetic gene was introduced into tobacco chloroplast which codes for a *C4V3* protein. It was shown that this protein can stimulate both systemic and mucosal immunity in BALB/c mice (Rubio-Infante et al., 2012).

Among bacterial agents, cholera toxin subunit B (CTB) was successfully expressed in chloroplasts in 2001 by Daniell, Lee, Panchal, and Wiebe (2001a), and it was found to bind to the GM1 receptor of intestinal membrane. In recent studies, Dreesen, Charpin-El Hamri, and Fussenegger (2010) reported the expression of a fusion protein in which the fibronectin binding domain from *Staphylococcus aureus* is fused with CTB with 80% immunogenic response. A heat liable toxin after fusion with heat stable toxin was produced by Rosales and Mendoza which induced both humoral and mucosal immunity, and Abs against both toxins were produced which successfully protected mice from infection of cholera toxin. Similarly, anthrax protective antigen with 18% TSP, *Borrlia burgfdorferi* (lyme disease) outer surface lipoprotein A with approximately 29% TSP, *Yersinia pestis* (plague) F1-V antigen with 15% TSP, and multiepitope DPT fusion protein with 0.8% TSP have been successfully expressed in tobacco chloroplasts (Lössl & Waheed, 2011).

Protozoan antigens have also been expressed in chloroplasts of tobacco and lettuce and their expression level was checked using antigen specific antibody titers. Gal/GalNAc lectin (LecA) of *Entamoeba histolytica*, which is the causative agent for amebiasis, has been successfully expressed with TSP accumulating up to 6.3% of total TSP with elevated IgG titers (Daniell et al., 2009). Malarial antigens (AMA1 and MSP1) fused with CTB have also been expressed in both tobacco and lettuce. The maximum level of CTB-AMA1 and CTB-MSP1 in tobacco was recorded as 14% and 10% of total TSP accumulation, respectively, which completely prevented further parasitic proliferation when administered in mice (Dreesen et al., 2010).

Autoantigens for diabetes type 1 have also been synthesized using chloroplasts from both tobacco (16% TSP) and lettuce (2.5% TSP), when CTB−proinsulin fusion protein (CTB-Pins) was orally administered. Nonobese mice after immunization showed a decline in insulitis. The expressions of IL-4 and IL-10, which are immunosuppressive in nature, were significantly increased (Ruhlman, Ahangari, Devine, Samsam, & Daniell, 2007).

Another advantage of using chloroplast-based vaccines is its use as a commercial edible plant for the production of biopharmaceutical proteins. This eliminates the need for expensive fermentation, extraction, storage, and transport costs, whereby proteins can be saved at ambient temperature and conditions for a longer period of time.

Chloroplast-derived vaccines have many new vistas for methods of vaccine production. Low cost vaccines are urgently required to combat infectious diseases, especially in third world countries. Chloroplast expression systems designed utilizes low economy, it is preferred for biosafety and large-scale production. Thus vaccine expression in chloroplasts requires a rational approach with numerous research groups in order to select preferred antigens.

18.4 Plant transgenomics: a way forward

A review of literature from the last few years highlights that plant transgenomics has originated as a separate field for the development of transgenic plants to produce biopharmaceutical products, vaccines, antibodies, hormones, enzymes, and drugs (Daniell, Streatfield, & Wycoff, 2001b; Price, 2003). An important aspect is the selection of plant species and organs for the introduction of transgenes. A number of plants, including tobacco species, provide secondary packaging of some human proteins such as secretory immunoglobulins A, which display partial humanization of *N*-glycans (Budzianowski, 2009). This effect is unattainable with a prokaryotic system, such as a bacterial-based expression system (Werner, 1999). For instance, the postharvest tobacco leaf system provides protein processing through posttranslational modification and endomembrane targeting (Cramer, Boothe, & Oishi, 2000). Furthermore, a plant-based expression system offers additional properties to vaccines, for example, the introduction of starch naturally present in plants known as granule bound starch synthase (GBSS) to malarial vaccines provides the polysaccharide matrix required for vaccine stability (Dauvillée et al., 2010).

Some drugs have already met different phases of clinical trials, some of the plant-based protein products, such as carrot cell-derived glucocerebrosidase hormone for Gaucher's patient, are already available on the personalized medication demand of the patient and hirudin is being commercially produced in Canada (Giddings et al., 2000). A new cattle disease vaccine met USDA approval in 2006 and is now being produced for commercial purposes. Hence, there is clearly a potential for drug and vaccine production in this area of research.

It was also found that the extensive purification, extraction, fermentation, and filtration processes that are required in microbial or animal system-derived biological agents are carried out with ease when manufacturing plant-derived drugs, which could down-scale the processing requirements and allow for the drugs' commercialization at a feasible cost (Xiao, Bai, Liu, & Wang, 2003).

Safe and effective vaccines with less adverse effects are prime targets in modern vaccines development (Bellanti, 2006). Vaccines which include subunit vaccines, adjuvant production, oral and chimeric proteins can be developed using plant transgenic systems. Furthermore, the potential for developing edible vaccines via the use of transgenic plants and fruits can allow herd immunization for the masses, which holds a great attraction for researchers. Edible vaccines, a novel plant expression system, can be manufactured on a large scale and delivered efficiently to the targeted individuals, when it is included in a staple crop or fruit such as a potato or banana (Tiwari, Verma, Singh, & Tuli, 2009). Upscaling of products via agriculture is another distinguishing characteristic that can help overcoming capital and shortage problems associated with drug and vaccines production in developing countries (Ulmer, Valley, & Rappuoli, 2006).

A plant-based expression system has some limitations, such as difficulty in developing a chloroplast expression system in important plant species, for example, *Arabidopsis*, which can act as an ideal plant model for chloroplast functional genomics (Dhingra & Daniell, 2006). The synthesis of such a system is important as it induces the formation of a plant efflux-based detoxification system naturally, which then helps multidrug, such as alkaloids and antibiotics, release from a plant system (Li, He, Pandey, Tsuchiya, & Luan, 2002). Another important aspect that needs to be addressed is provision of easy purification, extraction, and mass-scale production of recombinant proteins (Fischer, Stoger, Schillberg, Christou, & Twyman, 2004). Tissue culture techniques, including suspension-cultured plant cells of *N. tabacum* and *A. thaliana*, can be employed for the recombinant protein production since they counter issues, such as downstream processing and ease of cultivation, purification, and extraction, that are unattainable with transgenic plants (Plasson et al., 2009).

It was found that plant-based *N*-glycosylation was proven to be immunogenic in some studies and there is a need to develop plant-based system to express nonimmunogenic recombinant proteins in humanized glycan patterns (Decker & Reski, 2008). An alternative could be a moss *Physcomitrella* tissue culture system (Decker & Reski, 2012).

There is a need to address regulatory concerns that are associated with the clinical large-scale production of safe biopharmaceutics in transgenic plants (Miele, 1997). Policies have to be made for the containment of waste products produced by transgenic plants and monitoring by regulatory authorities (van der Laan et al., 2006). Hence, the risk associated with the environmental release of such transgenic plants has to be addressed for social acceptance of plant-based biopharmaceuticals, vaccines, and therapeutic drugs production, which can only be then commercialized on a mass scale (Peterson & Arntzen, 2004).

18.5 Conclusions

In comparison with conventional methods for vaccine production, plant-derived vaccines (PDVs) have clear advantages, such as the low cost, efficacy, safety, and the ease of development (Guan et al., 2013). When tested for safety and immunogenicity of such vaccines, it was found that PDVs were also eliciting high immunogenecity in vaccinated individuals during phase 1 clinical studies. Also, the ease of scaling up such vaccines is easier through the commercial level of crop cultivation (Tiwari et al., 2009). The heat-treatment for Ag derived from such vaccines is also dependent on Ag type, hence the concept of "cooked vaccine" is also appealing to the researchers. The idea of edible vaccines also eliminates the need for a "cold storage" in vaccine supply to the general population, hence countering the effective monitoring of a vaccination program against an infectious disease, while maintaining the stability of a vaccine (Sala et al., 2003). It is thus generally accepted that vaccines derived from plants can help in giving a new dimension to the vaccination and immunization programs carried out on a mass scale while effectively reducing the cost.

At present, biopharmaceuticals and vaccines derived from transgenic plants are evolving as an independent field of biotechnology for biopharmaceutical production as the ease of expression, purity, extraction, and filtration makes the PDPs highly attractive for the researchers. However, for the commercialization of antibodies, drugs, nutraceuticals, diagnostic proteins, and large-scale protein synthesis, a lot of research still has to be pursued. Safety, efficacy, public

acceptance, and ethical issues have to be considered before plant biotechnology can be established as an independent tool for pharmaceutical applications. However, some of the medications that are already in their clinical trials show promising results for development and commercialization, which can change the prospects for pharmaceutical biotechnology in the near futures if the initial problems associated with it are addressed correctly.

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Chapter 19

Secondary metabolites from endangered *Gentiana, Gentianella, Centaurium,* and *Swertia* species (Gentianaceae): promising natural biotherapeutics

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19.1 Introduction

The plant kingdom represents an inexhaustible source of unique compounds. In recent time, there has been a constant demand for plants as a potential sources of novel compounds with medical properties. However, of the estimated 400,000−500,000 plant species around the world, only a small percentage have been investigated for their biological and pharmacological activities (Pan et al., 2013). According to the latest data around 6% of higher plants have been studied for their pharmacological potential, and only 15% have been evaluated for phytochemicals in general (Espinosa-Leal, Puente-Garza, & García-Lara, 2018). The curative value of plants depends on the presence of biologically active compounds (Pan et al., 2013). These compounds, also known as secondary metabolites or phytochemicals, are products of plant secondary metabolism.

19.2 Secondary metabolites

Plant secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of the plant organism and are not synthesized by metabolic pathways common to all plant species (Fraenkel, 1959). They are derived by specified biosynthetic pathways from primary metabolites and intermediates. Unlike primary metabolites found throughout the plant kingdom, secondary metabolites are often limited to particular families, genera, or even species. Since they have limited distribution in taxonomic groups, these compounds are very useful as chemotaxonomic markers.

Secondary metabolites play a diverse functional role in plant protection against herbivores and microbial pathogens (viruses, fungi, and bacteria) (Ballhorn, Kautz, Heil, & Hegeman, 2009). Some of them are important for plant survival; others are involved in defense against abiotic stress or adaptation of plants to their natural environment. Also, secondary metabolites are essential signaling molecules in plant communication with other organisms (Schäfer & Wink, 2009). Plants produce a high diversity of secondary metabolites which may be classified into three chemically different classes: terpenoids, phenolics, and alkaloids (Mazid, Khan, & Mohammad, 2011).

The main secondary metabolites of *Gentiana*, *Gentianella*, *Centaurium*, and *Swertia* are phenols (xanthones and *C*-glucoflavonoids) and terpenoids (iridoids) and this chapter will focus on their biopharmacological activity.

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19.2.1 Terpenoids

Terpenoids or terpenes (carotenoids, sterols, cardiac glycosides, and plant volatiles) represent the largest group of secondary metabolites with more than 40,000 structurally diverse compounds (Bohlmann & Keeling, 2008). All terpenoids are derived through a common biosynthetic pathway from acetyl-CoA or glycolytic intermediates (Grayson, 1987). They are synthesized by condensation of isoprene units (C5) and are classified according to five-carbon units present in the core structure into: half terpenes or hemiterpenes (terpenes with 5-C), monoterpenes (10-C), sesquiterpenes (one and a half terpenes; 15-C), diterpenes (20-C), sesterterpenes (two and a half terpenes; 25-C), triterpenes (30-C), tetraterpenes (40-C), and polyterpenes (above 40-C, $(C5)_n$) (Mahmoud & Croteau, 2002). Some terpenes, such as the group of phytohormones gibberellins and brassinosteroids, have well-characterized functions in plant growth. However, most terpenes play a diverse functional role in plant protection against pathogens. Numerous terpenes act as phytoalexins, insecticidal and repellent compounds (Ahmed et al., 2017; Picman, 1986). For the sessile plants, volatile terpenoids provide communication with other organisms such as adjacent plants, pollinators, and herbivores, via air-borne info-chemicals (Dudareva, Negre, Nagegowda, & Orlova, 2006).

19.2.1.1 Iridoids

Iridoids belong to terpenoids and are found in numerous plant families usually as glycosides (Dinda, Debnath, & Harigaya, 2007). Structurally, iridoids are monoterpenoids whose basic structure is formed as a result of *cis*-fusion of cyclopentane and oxygen-containing heterocyclic rings (Nangia, Prasuna, & Bheema Rao, 1997). The cleavage of the cyclopentane ring of iridoids produces secoiridoids which are typical iridoid glucosides found in the Gentianaceae species (Dinda et al., 2007). The biosynthetic pathway universally present in Gentianaceae leads from iridodial via deoxyloganic acid and loganin or loganic acid to secoiridoids sweroside (SWS), swertiamarin (SWM), and gentiopicroside (GP). At least one of these compounds, responsible for the bitter character of plants, is found in practically all Gentianaceae species (Jensen & Schripsema, 2002). The iridoids found in endangered Gentianaceae species from *Gentiana*, *Gentianella*, *Centaurium*, and *Swertia* genera are listed in Tables 19.1 and 19.2.

19.2.2 Phenolics

Plant phenolics (flavonoids, phenolic acids, tannins, lignans, coumarins, lignins, stilbenes, and xanthones) are compounds characterized by the presence of a phenol group composed of an aromatic ring bearing one or more hydroxyl groups. This large chemically heterogeneous group of secondary metabolites produced by almost all plants is distributed in vegetables, fruits, teas, cereals, spices, and other edible plants, well-known in human nutrition as health promoters (Shetty, 2007). In plants, phenolics are synthesized via the common biosynthetic pathway and precursors derived from shikimate-phenylpropanoid and acetate-malonate pathways. The most abundant phenolic compounds derived from the phenylpropanoid-acetate-malonate pathway are flavonoids and isoflavonoids, the main constituents of fruits and legumes (Strack, 1997). As a result of hydroxylation, methylation, isoprenylation, dimerization, and glycosylation of the substituents in the aromatic nucleus, flavonoids are subdivided into several categories: flavonols, flavones, flavanols, isoflavones, and antocyanidins (Shetty, 2007). Considering their chemical diversity, phenolics play a variety of roles in the plant. Most of them are involved in the plant defense mechanism and protect plants from herbivores, pathogenic insects, bacteria, and fungi. Their high concentration imparts fungal resistance (Parr & Bolwell, 2000) and shields plants from UV radiation and cold stress (Harborne & Williams, 2000). The most important function of phenolic compounds is their antioxidant activity. Since they consist of an aromatic ring and hydroxyl groups, phenolics can act as effective antioxidants due to their ability to quench free electrons and chelate metal ions responsible for generating free radicals (Rice-Evans, Miller, & Paganga, 1997). Hence, plant phenolics can protect biosystems against free radicals attack, due to their ability to donate hydrogen from hydroxyl groups attached along the benzene ring and terminate free radical oxidation of lipids and other biomolecules (Foti, 2007). Therefore phenolic phytochemicals from food plants play a vital role as natural antioxidants in the prevention of disease and human health.

19.2.2.1 Xanthones

Xanthones (dibenzo-γ-pirone) belong to the class of oxygenated heterocycles and their biological and pharmacological activities are well-known in phytomedicine and medicinal chemistry (Pinto, Sousa, & Nascimento, 2005). The majority of xanthones occur in two plant families—Guttiferae and Gentianaceae—which still remain a principal source of xanthone compounds (Jensen & Schripsema, 2002). Distribution and oxygenation pattern of naturally occurring xanthones

TABLE 19.1 Secondary metabolites from endangered Gentianaceae species according to IUCN list.

Plant species	IUCN category	Secondary metabolites		References
Gentiana acaulis L. Synonyms: *G. kochiana* E.P.Perr.&Song. *G. excisa* C.Presl *G. grandiflora* Lam. *G. vulgaris* (Neilr.) Beck *Gentianusa acaulis* (L.) Pohl *Ericala alpina* G.Don *Ciminalis acaulis* Borkh. *Ciminalis acaulis* Moench *Ciminalis grandiflora* Mayrh. *Ciminalis longiflora* Moench	**LC**	**Secoiridoids**	Sweroside Swertiamarin Gentiopicrin	Šavikin et al. (2015)
		Xanthones	Gentiacaulenin Gentiakochianin Decussatin Isogentiacaulein 2,4,5-Trihydroxy-1-methoxyxanthon Gentiacauloside Decussatin-1-*O*-prim. Gentiokochianin-7-*O*-prim. Gentiacaulein-1-*O*-prim.	Peres, Nagem, and de Oliveira (2000), Plouvier, Massicot , and Rivaille (1967), Šavikin et al. (2015)
Gentiana kurroo Royle	**CR**	**Secoiridoids**	Sweroside Swertiamarin Gentiopicroside Amaroswerin Morroniside Gentiopicrin	Skinder, Ganai, and Wani (2017), Wani et al. (2013)
		Xanthones	Mangiferin Norswertianolin Bellidifolin-8-*O*-glc Gentisin Isogentisin Gentioside	Skinder et al. (2017), Wani et al. (2013)
Gentiana pannonica Scop. Synonyms: *Coilantha pannonica* G.Don *Gentiana semifida* Hoffmanns. ex Rchb. *Gentiana punctata* Jacq. *Gentiana purpurea* Schrank	**NT**	**Secoiridoids**	Swertiamarin Gentiopicrin Sweroside Amarogentin Amaropanin Amaroswerin	Wagner and Vasirian (1974)
		C-glucoflavones	Isoorientin Isovitexin Isoorientin-4′-*O*-glc Isovitexin-4′-*O*-glc	Hostettmann, Duc, Goetz, and Jacot-Guillarmod (1975), Pan, Zhao, Zhang, Li, and Wang (2016)
Gentiana punctata L. Synonyms: *Gentiana punctata* Jacq. *Gentiana punctata* Vill.	**LC**	**Secoiridoids**	Swertiamarin Gentiopicrin Sweroside Amarogentin Gentioflavoside	Popov and Marekov (1971), Šavikin et al. (2015)
		Xanthones	Gentisein Gentisin	Jensen and Schripsema (2002), Šavikin et al. (2015)
		C-glucoflavones	Isoorientin Isovitexin Isoorientin-4′-*O*-glc Isovitexin-4′-*O*-glc	Hostettmann et al. (1975), Pan et al. (2016)
Gentiana purpurea L. Synonyms: *Gentiana purpurea* Vill. *Gentiana purpurea* Walter *Gentiana purpurea* Schrank	**LC**	**Secoiridoids**	Amaropanin Amarogentin Amaroswerin Gentiopicroside Gentiolactone	Jensen and Schripsema (2002), Suhr, Arends, and Jensen (1978)
		C-glucoflavones	Isovitexin Isoorientin Isosaponarin	Hostettmann et al. (1975), Pan et al. (2016)
Gentianella rupicola (Kunth) Holub	**LC**	**Secoiridoids**	Gentiopicroside	Vidari and VitaFinzi (2010)

(*Continued*)

TABLE 19.1 (Continued)

Plant species	IUCN category	Secondary metabolites		References
Centaurium erythraea Rafn Synonyms: *Centaurium latifolium* (Sm.) Druce *Erythraea centaurium* (L.) Pers.	**LC**	**Xanthones**	Eustomin Demethyleustomin 1,5-Hydroxy-3-methoxyxanthone Decussatin 1-Hydroxy-3,5,6-trimethoxyxanthone 1,3,5-Trihydroxyxanthone Swerchirin	Aberham, Pieri, Croom, Ellmerer, and Stuppner (2011), Peters, Schmidt, and Beerhues (1997), Trifunović-Momčilov , Krstić-Milošević, Trifunović, Podolski-Renić, Pešić, and Subotić (2016), Valentão et al. (2002)
		Secoiridoids	Sweroside Deacethylcentapicrin Centapicrin Swertiamarin Gentiopicroside Gentioflavoside Secologanin Centauroside	Do, Popov, Marekov, and Trifonov (1987), Sakina and Aota (1976), Takagi, Yamaki, Yumioka, Nishimura, and Sakina (1982), van der Sluis (1985), van der Sluis and Labadie (1981)
Centaurium pulchellum (Sw.) Druce *Synonyms:* *Centaurium meyeri* (Bunge) Druce *Eryhtraea morierei* *Erythraea pulchellum*	**LC**	**Secoiridoids**	Gentiopicroside Sweroside Swertiamarin	Krstić et al. (2003), van der Sluis (1985), van der Sluis and Labadie (1981)
		Xanthones	Decussatin Swertiaperenine Swerchirin Demethyleustomin	Krstić et al. (2003), Miana and Al-Hazimi (1984)
Centaurium somedanum M. Laínz Synonym: *Centaurium chloodes* subsp. *somedanum* (Laínz) Romero.	**VU**	**Secoiridoids**	Gentiopicroside Sweroside Swertiamarin Decentapicrin A	van der Sluis (1985)
Swertia iberica Fisch. ex Boiss Synonyms: *Swertia balansae* Boiss. *Swertia stigmantha* K.Koch	**LC**	**Xanthones**	Swertiaperenine Decussatin Gentiakochianin Norswertinin Isogentiakochianin Swertiaiberin	Denisova et al. (1980)
Swertia longifolia Boiss. Synonyms: *Swertia aucheri* Boiss. *Swertia persica* Griseb.	**LC**	**Secoiridoids**	Swertiamarin	Denisova et al. (1980)
		Xanthones	1,5-Dihydroxy-3-methoxy-6-*O*-prim. 8-Hydroxy-3,5-dimethoxy-1-*O*-prim. Isobellidifolin Bellidin Gentisein Gentiacaulein Swerchirin Swertiaperenine	Hajimehdipoor, Dijoux-Franca et al. (2006b), Hajimehdipour, Amanzadeh, Sadat Ebrahimi, and Mozaffarian (2003)

IUCN category: *LC*, least concerned; *NT*, near threatened; *VU*, vulnerable; *EN*, endangered; *CR*, critically endangered.

have a high chemotaxonomic significance since they are not universal in the Gentianaceae. The diversity and grade of substitution of xanthones, as well as the oxygenation pattern, are characteristic and generally uniform within the genera (Jensen & Schripsema, 2002; Mészáros, 1994).

Naturally occurring xanthones are usually classified into six major groups: simple oxygenated xanthones, xanthones glycosides, prenylated xanthones, xanthonolignoids, bisxanthones, and miscellaneous xanthones (Negi, Bisht, Singh,

TABLE 19.2 Secondary metabolites from the protected and strictly protected species in the Republic of Serbia.

Plant species	Secondary metabolites		Plant organ	References
Gentiana asclepiadea L.[a]	**Secoiridoids**	Loganic acid Secologanosid Swertiamarin Gentiopicrin	Aerial parts	Šavikin et al. (2015)
		Sweroside Loganic acid Swertiamarin Gentiopicrin	Roots	Šavikin et al. (2015)
	Xanthones	Mangiferin Gentiacauloside	Aerial parts	Mihailović et al. (2013), Šavikin et al. (2015)
	C-glucoflavones	Isoscoparin Isovitexin Isoorientin Isosaponarin-*O*-glc	Aerial parts	Šavikin et al. (2015)
		Isosaponarin-*O*-glc Isoorientin-3′-*O*-glc	Roots	Šavikin et al. (2015)
Gentiana cruciata L. subsp. *cruciata*[a]	**Secoiridoids**	Swertiamarin Gentiopicrin Sweroside Loganic acid	Aerial parts	Mihailović et al. (2015)
		Swertiamarin Gentiopicrin Sweroside Loganic acid	Roots	Mihailović et al. (2015)
	C-glucoflavones	Vitexin Isovitexin-glucoside Isovitexin 4′,7-diglucoside	Aerial parts	Mihailović et al. (2015)
		Vitexin Orientin	Roots	Mihailović et al. (2015)
Gentiana dinarica Beck.[b]	**Secoiridoids**	Loganic acid Secologanosid Swertiamarin Gentiopicrin Sweroside	Leaves	Krstić et al. (2004), Šavikin et al. (2015)
		Swertiamarin Gentiopicrin Sweroside Amarogentin	Roots	Krstić et al. (2004)
	Xanthones	Norswertianin-1-*O*-glc Norswertianin-1-*O*-prim. Norswertianin-8-*O*-prim.	Roots	Krstić et al. (2004)
	C-glucoflavones	Isoorientin-3′-*O*-glc Isoorientin Isovitexin	Leaves	Krstić et al. (2004)
		Isoorientin Isovitexin	Roots	Krstić et al. (2004)

(*Continued*)

TABLE 19.2 (Continued)

Plant species	Secondary metabolites		Plant organ	References
Gentiana kochiana Perr. and Song. Synonym: *G. acaulis*[b]	**Secoiridoids**	Swertiamarin Gentiopicrin Sweroside	Leaves	Šavikin et al. (2015)
		Loganic acid Swertiamarin Gentiopicrin Sweroside	Roots	Šavikin et al. (2015)
	Xanthones	Gentiacaulenin Gentiakochianin Decussatin Gentiacaulenin-1-*O*-prim. Gentiacaulenin-1-*O*-glc Decussatin-1-*O*-prim. Gentiakochianin-7-*O*-prim.	Leaves	Peres et al. (2000), Rivaille et al. (1969), Šavikin et al. (2015)
		Gentiacaulenin Gentiakochianin-1-*O*-prim. Gentiacaulenin-1-*O*-glc Decussatin-1-*O*-prim. Gentiakochianin-7-*O*-prim.	Roots	Šavikin et al. (2015)
	C-glucoflavones	Isovitexin Isoorientin-3′-*O*-glc	Leaves	Šavikin et al. (2015)
Gentiana lutea L. subsp. *symphyandra* (Murb.) Hayek[a]	**Secoiridoids**	Loganic acid Secologanosid Swertiamarin Gentiopicrin Sweroside Amarogentin Amaropanin Amaroswerin	Underground organs	Šavikin et al. (2015)
		Eustomorussid Secologanosid Loganic acid Septemfidoside Swertiamarin Gentiopicrin Sweroside	Leaves	Šavikin et al. (2015)
	Xanthones	Gentioside Gentioside isomer Isogentisin	Underground organs	Šavikin et al. (2015)
		Mangiferin Gentioside Isogentisin	Leaves	Bellmann and Jacot-Guillarmod (1973), Menković, Šavikin-Fodulović and Savin (2000)
	C-glucoflavones	Isosaponarin Isoorientin Isovitexin Isoorientin-4′-*O*-glc Isovitexin 4′-*O*-glc	Leaves	Bellmann and Jacot-Guillarmod (1973), Hostettmann, Bellmann, Tabacchi, and Jacot-Guillarmod (1973), Kušar , Šircelj, and Baričevič (2010)

(*Continued*)

TABLE 19.2 (Continued)

Plant species	Secondary metabolites		Plant organ	References
Gentiana nivalis L.[b]	**Xanthones**	Decussatin Gentiacaulein Swertianin Mangiferin	Aerial parts	Hostettmann and Jacot-Guillarmod (1977)
	C-glucoflavones	Isoorientin Isovitexin Isoorientin-3′-*O*-glc	Aerial parts	Hostettmann and Jacot-Guillarmod (1974, 1977)
Gentiana pneumonanthe L. subsp. *pneumonanthe*[a]	**Secoiridoids**	Swertiamarin Gentiopicrin Sweroside	Aerial parts, roots	Popović et al. (2019)
	Xanthones	Mangiferin	Aerial parts	Popović et al. (2019)
	C-glucoflavones	Isoorientin Isovitexin	Aerial parts	Popović et al. (2019)
Gentiana punctata L.[a]	**Secoiridoids**	Loganic acid Secologanosid Swertiamarin Gentiopicrin Sweroside Amarogentin	Roots	Menkovic et al. (1998), Šavikin et al. (2015)
		Eustomosid Swertiamarin isomer Loganic acid Secologanosid Swertiamarin Gentiopicrin Sweroside	Aerial parts	Menkovic et al. (1998), Šavikin et al. (2015)
	Xanthones	Gentioside Isogentisin	Roots	Šavikin et al. (2015)
	C-glucoflavones	Isosaponarin Isoorientin Isovitexin Isoscoparin Isoorientin-3′-*O*-glc Isoorientin-3′-*O*-glc-isomer	Aerial parts	Šavikin et al. (2015)
Gentianella albanica (Jáv.) J. Holub[a]	**Secoiridoids**	Swertiamarin Gentiopicrin	Aerial parts, roots	Krstić-Milošević et al. (2015)
	Xanthones	Demethylbellidifolin Bellidifolin Corymbiferin Demethylbellidifolin-8-*O*-glc Bellidifolin-8-*O*-glc Corymbiferin-1-*O*-glc Veratriloside Lanceoside	Roots	Krstić-Milošević et al. (2015)
		Demethylbellidifolin Bellidifolin Corymbiferin Demethylbellidifolin-8-*O*-glc Bellidifolin-8-*O*-glc Corymbiferin-1-*O*-glc Lanceoside	Aerial parts	Janković, Krstic, Savikin-Fodulovic, Menkovic, and Grubisic (2005), Krstić-Milošević et al. (2015)
	C-glucoflavones	Isoorientin Swertisin	Aerial parts	Krstić-Milošević et al. (2015)

(*Continued*)

TABLE 19.2 (Continued)

Plant species	Secondary metabolites		Plant organ	References
Gentianella ciliata (L.) Borkh. subsp. *ciliata*[a]	**Xanthones**	Gentiakochianin Gentiacaulein Isogentiacaulein Decussatin Swertiaperenin	Aerial parts	Krstić-Milošević et al. (2015)
Centaurium erythraea Rafin. subsp. *erythraea*[a]	**Secoiridoids**	Sweroside Gentiopicrin Swertiamarin	Aerial parts, roots	Aberham et al. (2011), Šiler et al. (2012)
	Xanthones	Eustomin Demethyleustomin 1,5-Hydroxy-3-methoxyxanthone 1-Hydroxy-3,7,8-trimethoxyxanthon 1-Hydroxy-3,5,6-trimethoxyxanthone	Aerial parts	Trifunović-Momčilov et al. (2016), Valentão et al. (2002)
Swertia punctata Baumg.[b]	**Secoiridoids**	Secologanoside Loganic acid Septemfidosid Swertiamarin Gentiopicrin Sweroside Isosapoarin	Aerial parts	Menković et al. (2002), Šavikin et al. (2015)
	Xanthones	Bellidifolin Demethylbellidifolin Swertianolin Mangiferin Swerhirin	Aerial parts	Menković et al. (2002), Šavikin et al. (2015)
		Isobellidifolin Swerhirin Isobellidifolin-3-*O*-prim. Isoswertianin Methylswertianin Methylswertianin-1-*O*-gentiobioside	Roots	Menković et al. (2002), Šavikin et al. (2015)
	C-glucoflavones	Isoorientin Isovitexin Isoorientin-2″-*O*-glc	Aerial parts	Menković et al. (2002), Šavikin et al. (2015)

[a]*Protected in the Republic of Serbia.*
[b]*Strictly protected in the Republic of Serbia.*

Rawat, & Joshi, 2013; Peres and Nagem, 1997). According to the degree of oxygenation, the simple oxygenated xanthones are subdivided into non-, mono-, di-, tri-, tetra-, penta-, and hexaoxygenated compounds, where substituents are hydroxy, methoxy, or methyl groups.

In the Gentianaceae, xanthones occur as simple oxygenated xanthones (monooxygenated, trioxygenated, tetraoxygenated and hexaoxygenated) and xanthone glycosides. Xanthone glycosides appear as *C*- and *O*-glycosides and are isolated predominantly from species of the Gentianaceae and Polygalaceae. The carbon−carbon (C−C) bond between the xanthone nucleus and sugar moiety is characteristic for xanthone-*C*-glycosides, while xanthone-*O*-glycosides have classic glycosidic binding where sugar is attached by carbon−oxygen (C−O) bond. Xanthone-*O*-glycosides are very frequent among the Gentianaceae species. More than 20 *O*-glycosides have been found and most of them are monoglycosides. Conversely, the natural occurrence of xanthone-*C*-glycosides within the Gentianaceae is occasional. Overall, only seven xanthone-*C*-glycosides are isolated from Gentianaceae, including mangiferin and isomangiferin as the most common (Negi et al., 2013).

19.2.2.1.1 Biosynthesis of xanthones

Xanthones in higher plants are derived by a mixed shikimate-acetate pathway. Studies on *Garcinia mangostana* (Bennett & Lee, 1988) and *Centaurium erythraea* (Beerhues, 1996) showed that *m*-hydroxy benzoic acid is the principal precursor of xanthone biosynthesis, indicating that a C_6-C_1 unit and three acetate units form an intermediate benzophenone (C_{13} skeleton). This reaction, catalyzed by benzophenone synthase, is a central step in xanthone biosynthesis. Benzophenone is subsequently converted to the corresponding xanthone by phenol oxidative coupling (Bennett & Lee, 1988). The enzyme xanthone synthase is responsible for various mechanisms of this intramolecular reaction, yielding a wide variety of oxidation patterns in xanthones of higher plants (Atkinson & Lewis, 1969). For instance, oxidation of the benzophenone at only one of the positions C2′ and C6′ can give 1,3,5,8- or 1,3,7,8-tetraoxyxanthones (Carpenter, Locksley, & Scheinmann, 1969). Notably, *C*-glycosylxanthones (such as mangiferin) have a distinct biosynthetic pathway from the other xanthones. They are originated from flavonoid-type C_6-C_3 unit (*p*-coumarate) and two acetate units, indicating a biogenetic relationship between flavonoids and xanthones (Bennett & Lee, 1988).

19.2.2.2 C-Glucoflavones

C-Glucoflavones are secondary metabolites widely distributed in monocots. In dicots, they are found mainly in the Leguminosae, Gentianaceae, and Asteraceae (Jensen & Schripsema, 2002). Only nine different compounds have been isolated so far. Like in xanthone-*C*-glucoside mangiferin, the sugar component in *C*-glucoflavones is attached to the flavonoid nucleus by C−C bond. Isovitexin and isoorientin, two biosynthetically primary compounds, are the most common *C*-glucoflavones in *Gentiana*. The members of *Swertia* genus contain mostly isovitexin and swertisin, while the species from *Gentianella* produce isoorientin and swertisin.

19.3 Gentianaceae

According to the recent comprehensive research, the family Gentianaceae comprises more than 1600 species classified into 87 genera including *Gentiana*, *Gentianella*, *Swertia*, and *Centaurium*. Considering its geographic distribution the Gentianaceae represents a cosmopolitan family. The most species grow in alpine or temperate habitats. They are mostly distributed in the mountain regions of Europe, North and South America, Central Asia, Northwest Africa, and East Australia (Struwe et al., 2002). Within the Gentianaceae, *Gentiana* is the largest genus comprising over 360 species. *Gentianella* comprises 250 species; *Swertia* includes 135 species, while *Centaurium* is smaller genus with 50 species. All these species are characterized by diversity in habitats, morphology, and anatomy. In the central region of the Balkan Peninsula (the Republic of Serbia and the Republic of Montenegro), 11 species of the genus *Gentiana* (*G. lutea*, *G. punctata*, *G. cruciata*, *G. asclepiadea*, *G. pneumonanthe*, *G. kochiana*, *G. dinarica*, *G. verna*, *G. tergestina*, *G. utriculosa*, and *G. nivalis*), six *Gentianella* species (*G. austiraca*, *G. bulgarica*, *G. crispata*, *G. praecox*, *G. axillaris*, and *G. ciliata*), and four species of the genus *Centaurium* (*C. erythraea*, *C. uliginosum*, *C. pulchellum*, and *C. tenuiflorum*) are found. *Swertia punctata* is the only species of the genus *Swertia* occurring in the Republic of Serbia, while *Swertia perennis* is found in the Republic of Montenegro (Šavikin et al., 2015). Plant species from *Gentiana* are spread all over the world, but most of them are distributed in moderate to alpine regions of Southeast Asia, Europa, and North America. All species are annual, biennial, or perennial herbs (Struwe et al., 2002). *Gentiana* species have been utilized as remedies in traditional medicine for centuries. The bitter tasting of the gentian herbal extracts originates from secoiridoids which were found to be responsible for beneficial effects in the treatment of gastro-complaints.

The genus *Gentianella* Moench comprises annual or biennial species growing in temperate or mountain habitats globally, while the center of origin is thought to be in the northern hemisphere of Eurasia. *Gentianella* was originally classified as a subgenus within the genus *Gentiana*. From the 1920s, *Gentianella* was classified as an individual genus (Krstić-Milošević, Vinterhalter, Janković, & Vinterhalter, 2015).

Centaurium species are distributed mainly in the Mediterranean region, and in North and Central America. A few species also occur in South America and temperate Asia and Australia (Struwe et al., 2002). They are known as the plant drug "Centaurii herba," which is a common ingredient of many commercial formulations recommended for the treatment of digestive problems. Common centaury (*C. erythraea* Rafn) is one of the most important pharmacological species from the *Gentianaceae* family (Šiler and Mišić, 2016). The main class of secondary metabolites of all *Centaurium* species is bitter secoiridoid glucosides.

Swertia species occur in Europe and in temperate zones of Asia, North America, and Africa, though they are concentrated in eastern Asia at an altitude of 1200−3600 m (Köhlein et al., 1991; Negi, Singh, & Rawat, 2011). *Swertia* includes both annual and perennial species. Species produce xanthones as the main class of natural compounds along with others such as triterpenoids, secoiridoids, and flavonoids.

19.4 The importance of biodiversity as a source of naturally derived bioactive molecules

The pharmacological properties of secondary metabolites from the Gentianaceae, as an important resource for drug development, have raised a great interest in the past few decades. The vast and versatile Gentianaceae pharmacological activity is based on their main constituents—xanthones, iridoids, and *C*-glucoflavones. Xanthones are present only in a few plant families, mainly in the Gentianaceae and Guttiferae, but also in Moraceae, Clusiaceae, and Polygalaceae. Within distinctive genera, substitution grade and oxygenation patterns of xanthones are exclusive and in most cases are uniform (Meszaros, de Laet, & Smets, 1996). Iridoids, primarily secoiridoid glucosides (swertiamarin, sweroside, or gentiopicroside), unlike xanthones, are found in all investigated species in the family Gentianaceae (Negi et al., 2013). Evidently, some natural products are abundant throughout the Gentianacaea, the others, like swertiaiberin, identified only in *Swertia iberica* (Denisova, Glyzin, Patudin, & Fesenko, 1980), are distinctive for particular species. The species are distinguished not only by a characteristic composition of secondary metabolites but also by their specific amount stored in various parts of the plant. Accordingly, any attempt to reproduce a chemical diversity of plant extracts during drug development fails precisely because of the complexity of crude extracts.

Overall, the decline of Gentianaceae species poses a high risk to the loss of an enormous diversity of un/identified and potentially bioactive compounds, thus the preservation of every single plant species is imperative. The leading causes of species endangerment, according to the first global analysis of plant biodiversity status, are climate change and habitat loss (Ibrahim et al., 2013). Climate change is a crucial factor in distribution shifts of plant species around the world. To a lesser extent, habitat loss due to the conversion of natural habitats to agriculture and spreading of invasive species represents a threat to the extinction of plants. Concurrently, uncontrolled overharvesting and inadequate collecting of medical plant affect endangerment of particular species. In the absence of a suitable preservation strategy, all the abovementioned lead to uncontrolled plant exploitation from nature and could result with species extinction.

Therefore efforts should be made both to preserve the plant populations and to elevate the level of knowledge for sustainable utilization of these plants. The two most important steps in medicinal plants preservation are in situ and ex situ conservation. In situ conservation of plant species includes restoration of the suitable habitat based on the biological characteristics and ecological traits according to the natural habitat (e.g., nature reserves, wild nurseries). Ex situ conservation is the conservation and maintenance of plant samples outside their natural habitat, either in the form of the whole plant, or as a seed, pollen, and tissue or cell culture. One of the most commonly used techniques for ex situ conservation is biotechnology based on plant tissue culture methods. It offers a rapid and suitable way for mass propagation of plant species; it is mostly used for commercial purposes. These applications cover utilization in bioreactors for mass production of bioactive compounds, especially secondary metabolites, biosynthesis of novel components, biotransformation of low-cost precursors into high-value pharmaceuticals and improvement of medicinal plants through genetic transformation (Zhou & Wu, 2006). Indeed, in vitro culture studies and propagation of plant species from the genera *Gentiana* (Vinterhalter, Krstić-Milošević, Janković, Milojević, & Vinterhalter, 2012; Vinterhalter, Mitić, Vinterhalter, Uzelac, & Krstić-Milošević, 2016), *Gentianella* (Janković et al., 2011; Vinterhalter, Janković, Šavikin, Nikolić, & Vinterhalter, 2008), *Swertia* (Chaudhuri, Pal, & Jha, 2007; Shailja, Kanwar, Soni, & Singh, 2017), and *Centaurium* (Subotić, Janković, Jevremović, & Grubišić, 2006) have been well elaborated and documented. However, these methods can be also used for the protection and reintroduction of endemic and endangered plants, as well as for fast propagation of rare and slowly growing plants (Vanisree et al., 2004). Numerous species belonging to Gentianaceae are endemic, characterized by low germination capacity, and endangered by excessive harvesting and unfavorable environmental influences, thus in vitro conservation could lead toward the preservation of the biological and genetic diversity of Gentianaceae species. Nevertheless, one should be extremely cautious for applying the abovementioned in vitro methods when it comes to species preservation since it is found that the level of some natural products fluctuates depending on habitat (Yang, Ding, Duan, & Liu, 2005).

This chapter will focus on pharmacological activities of distinctive secondary metabolites (xanthones and secoiridoids) from endangered, protected, and strictly protected species belonging to four Gentianaceae genera: *Gentiana*, *Gentianella*, *Centaurium*, and *Swertia*. To select endangered, among all species, we have used International Union for Conservation of Nature's (IUCN) Red List, the world's most comprehensive information source on the global conservation status of animal, fungi, and plant species. It is set upon precise criteria to evaluate the extinction risk of thousands of species and subspecies. According to IUCN, all taxa are divided into seven categories: least concerned (LC), near threatened (NT), vulnerable (VU), endangered (EN), critically endangered (CR), extinct in the wild (EW), and extinct (EX). Vulnerable, endangered, and critically endangered come within a category of threatened taxa. Detailed criteria based on reduction in population size over the last 10 years or three generations, whichever is the longer, geographic range in the form of extent of occurrence and/or area of occupancy, and population size are used to classify a

taxon into one of the listed categories (IUCN, 2012). The risk of extinction increases from LC to CR. For example, the widespread and abundant taxa are included in the LC category, while population size reduction of $\geq 90\%$ over the last 10 years or three generations qualifies taxa as CR. However, it is important to emphasize here that a taxon may require conservation action even if it is not listed as threatened. For the selection of protected and strictly protected species from the Republic of Serbia, we have used a list of endangered species defined by law of the Republic of Serbia.

All Gentianaceae species from four genera of interest (*Gentiana*, *Gentianella*, *Centaurium*, *Swertia*) listed at the IUCN site are compiled in Tables 19.1 and 19.3. Some of the species from the IUCN list currently belong to the LC category. The protected and strictly protected species in the Republic of Serbia are listed in Table 19.2. Secondary metabolites found in selected species are presented in Tables 19.1 and 19.2. Xanthones and secoiridoids, as compounds with the most interesting and significant bioactivities are cited separately (Tables 19.4–19.18 and 19.19–19.21).

In the following pages, up-to-date bioactivities of xanthones and typical secoiridoids (sweroside, swertiamarin, and gentiopicroside) found in endangered Gentianaceae species are explained in detail, with the aim of providing comprehensive insight into their pharmacological potential. Due to the low availability of plant material and thus limited amount of research regarding endangered plants, we have presented all bioactivities of the particular xanthone and secioridoid regardless of their origin. Mangiferin is not included in the survey since its bioactivity is comprehensively reviewed in numerous papers (Du et al., 2018; Imran et al., 2017; Saha, Sadhukhan, & Sil, 2016).

19.5 Pharmacological activities of xanthones

19.5.1 1-Hydroxy-3,5-dimethoxyxanthone

One of the major contributors to myocardial injury is the disturbed balance between reactive oxidative species (ROS) and antioxidant defense mechanisms (Kurian, Rajagopal, Vedantham, & Rajesh, 2016). Since the antioxidant potential of xanthones is well-known, the possible cardioprotective activity of 1-hydroxy-3,5-dimethoxyxanthone was evaluated in vitro and ex vivo (He & Xu, 2000; He, Xu, & Deng, 2000). The 1-hydroxy-3,5-dimethoxyxanthone and two other xanthones (swerchirin and decussatin) isolated from *Canscora lucidissima* (Gentianaceae) showed protective effect toward impairment of rat heart mitochondria induced with ascorbate and $FeSO_4$ in vitro (He & Xu, 2000) and in the anoxia/reoxygenation model in cultured neonatal rats myocardial cells (He et al., 2000). The investigated xanthones increased the membrane fluidity of damaged mitochondria, and decreased the lipid peroxidation and mitochondria swelling (He et al., 2000). In cultured myocardial cells exposed to anoxia/reoxygenation, in the presence of investigated xanthones the viability rate of myocardial cells and membrane fluidity were recovered, while lactate dehydrogenase release was reduced (He et al., 2000). The cardioprotective potential was additionally confirmed in vivo. Pretreatment with 1-hydroxy-3,5-dimethoxyxanthone from *C. lucidissima* prevented the ischemic reperfusion myocardial injury of rats heart exacerbated by activation of Na^+/H^+ exchange system (He et al., 2000). Further, an intravenous usage (1 mg/kg) of 1-hydroxy-3,5-dimethoxyxanthone and other xanthones isolated from *C. lucidissima*, before the left coronary artery ligation, substantially lowered the incidence and duration of ventricular arrhythmia, reduced all parameters of myocardial cells damage, elevating simultaneously superoxide dismutase (SOD) activity (He, Xu, & Peng, 1998). Hence, the protective effect of xanthones is most probably related to the attenuation of myocardial lipid peroxidation and the augmentation of SOD activity, thus confirming a correlation between cardioprotective and antioxidant abilities of investigated xanthones.

In order to investigate the potential anti-cancer properties of *Lomatogonium carinthiacum* (Gentianaceae), Jia et al. (Jia, Guo, Jia, & Sun, 2011) studied cytotoxic activities of chloroform, butanol, and water plant extracts against human lung adenocarcinoma A549, human erythroleukemia K562, and human cervical carcinoma HeLa cell lines. Among all three extracts, the xanthone-rich chloroform extract most robustly induced shrinkage and detachment of cells in culture. The half-maximal inhibitory concentration (IC_{50}) values against the A549, K562, and HeLa cell lines were 0.13, 0.76, and 0.61 mg/mL, respectively. HPLC analysis of chloroform extract revealed the presence of various xanthones, along with 1-hydroxy-3,5-dimethoxyxanthone, but further studies to determine which xanthones were responsible for the cytotoxic effect of the xanthone-rich extract had not been performed.

Cytotoxic activity of seven xanthones isolated, for the first time, from whole plant *Codonopsis ovata* (Campanulaceae) was investigated (Dar et al., 2016). The following metabolites, also present in genus *Gentiana*, were isolated and identified: 1-hydroxy-3,5-dimethoxyxanthone, swertiperenine, and gentiakochianin. Their activity against six human cancer cell lines—lung adenocarcinoma A549, prostate PC-3, colon HCT-116, breast MCF-7 and MDAMB-435, and brain SF-295 cancer—was determined by sulforhodamine B assay. The 1-hydroxy-3,5-dimethoxyxanthone exhibited toxic effect only against

TABLE 19.3 The endangered Gentianaceae species, according to IUCN list, with unidentified phytochemical content.

Plant species	IUCN category
Gentiana douglasiana Bong.	LC
Gentiana ligustica (R.Vilm. & Chopinet) Holub	LC
Gentiana rubricaulis Schwein. Synonyms: *Dasystephana grayi* (Kusn.) Britton *Gentiana grayi* Kusn.	LC
Gentiana sceptrum Griseb.	LC
Gentiana wangchukii E.Aitken & D.G.Long	LC
Gentianella androsacea J.S.Pringle	EN
Gentianella anglica (Pugsley) E.F.Warb.	Data deficient
Gentianella bohemica Skalický Synonym: *Gentianella gabretae* Skalický	VU
Gentianella cernua (Kunth) Fabris	LC
Gentianella crassulifolia (Griseb.) Fabris	VU
Gentianella fastigiata Fabris	VU
Gentianella flaviflora (Gilg) Fabris	EN
Gentianella foliosa (Kunth) Fabris Synonyms: *Gentiana androtricha* Gilg *Gentiana coarctata* Willd. ex Schult. *Gentiana stellarioides* Griseb. *Gentianella spruceana* Fabris *Gentianella stellarioides* (Griseb.) Fabris	LC
Gentianella fuscicaulis Fabris	EN
Gentianella gilioides (Gilig) Fabris	VU
Gentianella gracilis (Kunth) Fabris	EN
Gentianella griersonii E.Aitken & D.G.Long	LC
Gentianella hirculus (Griseb.) Fabris	EN
Gentianella hypericoides (Gilg) Fabris	VU
Gentianella hyssopifolia (Kunth) Fabris	VU
Gentianella jamesonii (Hook.) Fabris Synonyms: *Gentiana arcuata* Griseb. *Gentiana inflata* Griseb. *Gentiana jamesonii* Hook. *Gentiana pendula* Griseb.	EN
Gentianella limoselloides (Kunth) Fabris Synonym: *Gentiana peduncularis* Willd. ex Schult.	LC
Gentianella longibarbata (Gilg) Fabris	EN
Gentianella oellgaardii J.S.Pringle	VU
Gentianella polyantha J.S.Pringle	EN
Gentianella profusa J.S.Pringle	VU
Gentianella rupicola (Kunth) Holub	LC
Gentianella saxifragoides (Kunth) Fabris	VU
Gentianella splendens (Gilg) Fabris	LC

(*Continued*)

TABLE 19.3 (Continued)

Plant species	IUCN category
Gentianella sulphurea (Gilg) Fabris	VU
Centaurium candelabrum H. Lindb Synonym: *Centaurium pulchellum* subspecies *grandiflorum* (Batt.) Maire	LC
Centaurea leptophylla (K.Koch) Tchich.	CR
Centaurium sebaeoides (Griseb.) Druce	CR
Centaurium somedanum M. Laínz Synonym: *Centaurium chloodes* subsp. *somedanum* (Laínz) Romero.	VU
Swertia crossoloma Harry Sm.	LC
Swertia grandiflora Harry Sm.	LC
Swertia rosulata (Baker) Klack. Synonym(s): *Exacum rosulatum* Baker *Lomatogonium lubahnianum* (Vatke) Fernald *Pleurogyne lubahniana* Vatke *Swertia lubahniana* (Vatke) Engl.	NT

IUCN category: *LC*, least concerned; *NT*, near threatened; *VU*, vulnerable; *EN*, endangered; *CR*, critically endangered.

TABLE 19.4 Endangered Gentianaceae species containing 1-hydroxy-3,5-dimethoxyxanthone.

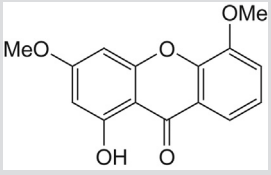
1-Hydroxy-3,5-dimethoxyxanthone	Plant species	References
	*Centaurium erythraea*	Aberham et al. (2011)

TABLE 19.5 Endangered Gentianaceae species containing mesuaxanthone A.

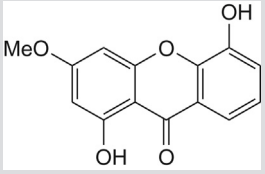
Mesuaxanthone A (1,5-dihydroxy-3-methoxyxanthone)	Plant species	References
	*Centaurium erythraea*	Aberham et al. (2011), Valentão et al. (2002)

TABLE 19.6 Endangered Gentianaceae species containing gentisein.

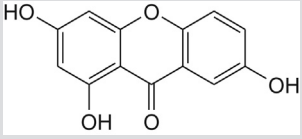
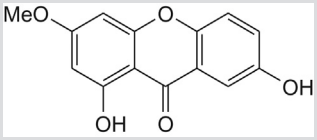
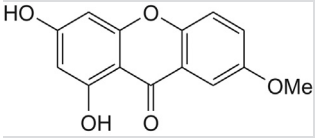
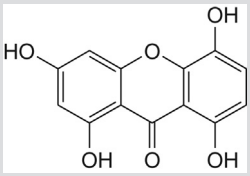
Gentisein (1,3,7-trihydroxy xanthone)	Plant species	References
	*Swertia longifolia*	Hajimehdipoor, Sadeghi et al. (2006)
	Gentiana punctata	Jensen and Schripsema (2002)
	Gentiana purpurea	Jensen and Schripsema (2002)

TABLE 19.7 Endangered Gentianaceae species containing gentisin and isogentisin.

Gentisin (1,7-dihidroxy-3-methoxyxanthone)	Plant species	References
	*Gentiana kurroo*	Wani et al. (2013)
Isogentisin (1,3-dihidroxy-7-methoxyxanthone)	**Plant species**	**References**
	*Gentiana kurroo*	Wani et al. (2013)
	Gentiana lutea[a]	Šavikin et al. (2015)
	Gentiana punctata[a]	Šavikin et al. (2015)

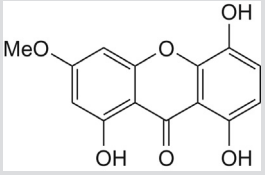
[a]*Protected in the Republic of Serbia.*

TABLE 19.8 Endangered Gentianaceae species containing demethylbellidifolin.

Demethylbellidifolin (1,3,5,8-tetrahydroxyxanthone, desmethylbellidifolin, bellidin)	Plant species	References
	*Swertia longifolia*	Hajimehdipoor, Sadeghi et al. (2006)
	Gentianella albanica[a]	Krstić-Milošević et al. (2015)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)

[a]*Protected in the Republic of Serbia.*

TABLE 19.9 Endangered Gentianaceae species containing bellidifolin.

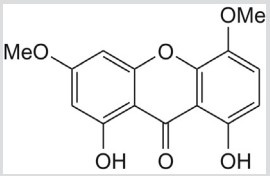
Bellidifolin (1,5,8-trihydroxy-3-methoxy xanthone)	Plant species	References
	*Centaurium erythraea*	Aberham et al. (2011)
	Gentianella albanica[a]	Krstić-Milošević et al. (2015)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)

[a]*Protected in the Republic of Serbia.*

A549 cell line with the IC_{50} value of 3 μM. Similarly, two other xanthones, exhibited cytotoxic potential only toward one cell line, suggesting that xanthones most probably possess cell-specific cytotoxic activity.

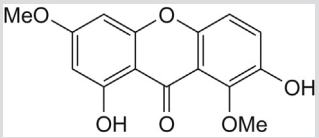
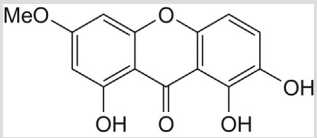
Among several different constituents of petroleum ether fraction of *Swertia chirayita*, 1-hydroxy-3,5-dimethoxyxanthone was identified (You et al., 2017). In this study, cytotoxic properties of 1-hydroxy-3,5-dimethoxyxanthone were not shown against human pancreatic SW1990 or BxPC-3 cancer cell lines, confirming that xanthone anti-tumor activity is cell-specific.

TABLE 19.10 Endangered Gentianaceae species containing swerchirin.

Swerchirin (1,8-dihydroxy-3,5-dimethoxyxanthone, methylbellidifolin)	Plant species	References
	*Centaurium erythraea*	Aberham et al. (2011)
	Swertia longifolia	Hajimehdipour et al. (2003)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)

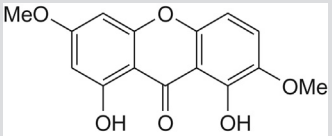
[a]*Protected in the Republic of Serbia.*

TABLE 19.11 Endangered Gentianaceae species containing gentiacaulein and gentiakochianin.

Gentiacaulein (1,7-dihidroxy-3,8-dimethoxyxanthone)	Plant species	References
	*Swertia longifolia*	Hajimehdipour et al. (2003)
	Gentiana acaulis	Peres et al. (2000), Šavikin et al. (2015)
	Gentiana nivalis[a]	Hostettmann and Jacot-Guillarmod (1977)
	Gentianella ciliata[a]	Krstić-Milošević et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)
Gentiakochianin (1,7,8-trihidroxy-3-methoxyxanthone, swertianin)	**Plant species**	**References**
	*Swertia iberica*	Denisova et al. (1980)
	Gentiana acaulis	Peres et al. (2000), Šavikin et al. (2015)
	Gentiana nivalis[a]	Hostettmann and Jacot-Guillarmod (1977)
	Gentianella ciliata[a]	Krstić-Milošević et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)

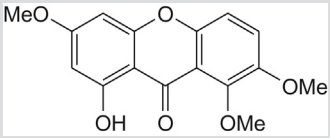
[a]*Protected in the Republic of Serbia.*

TABLE 19.12 Endangered Gentianaceae species containing swertiaperennine.

Swertiaperenine (1,8-dihydroxy-3,7-dimethoxyxanthone, swertiaperennin, methylswertianin)	Plant species	References
	*Swertia longifolia*	Hajimehdipour et al. (2003)
	Swertia iberica	Denisova et al. (1980)
	Gentianella ciliata[a]	Krstić-Milošević et al. (2015)
	Gentianella punctata[a]	Menković et al. (2002), Šavikin et al. (2015)

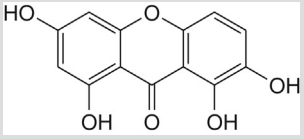
[a]*Protected in the Republic of Serbia.*

TABLE 19.13 Endangered Gentianaceae species containing decussatin.

Decussatin (1-hydroxy-3,7,8-trimethoxyxanthone)	Plant species	References
	*Centaurium erythraea*	Valentão et al. (2002)
	Swertia iberica	Denisova et al. (1980)
	Gentiana acaulis	Peres et al. (2000)
	Gentiana nivalis[a]	Hostettmann and Jacot-Guillarmod (1977)
	Gentianella ciliata[a]	Krstić-Milošević et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)

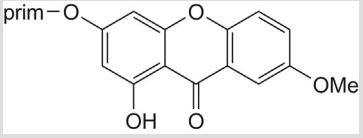
[a]*Protected in the Republic of Serbia.*

TABLE 19.14 Endangered Gentianaceae species containing norswertianin.

Norswertianin (1,3,7,8-tetrahydroxyxanthone)	Plant species	References
	*Swertia iberica*	Denisova et al. (1980)
	Gentiana dinarica[a]	Krstić, Janković, Aljančić, Šavikin-Fodulović, Menković, ad Milosavljević (2004)

[a]*Protected in the Republic of Serbia.*

TABLE 19.15 Endangered Gentianaceae species containing gentioside.

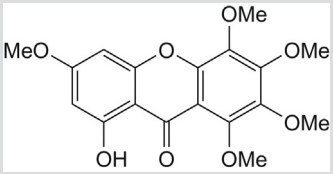
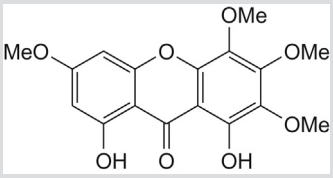
Gentioside (1-hydroxy-3-*O*-β-primeverosyl-7-methoxy xanthone)	Plant species	References
	*Gentiana kurroo*	Wani et al. (2013)
	Gentiana lutea[a]	Šavikin et al. (2015)
	Gentiana punctata[a]	Šavikin et al. (2015)

[a]*Protected in the Republic of Serbia.*

19.5.2 Mesuaxanthone A

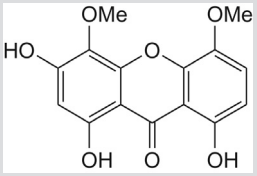
One of the earliest studies of mesuaxanthone A bioactive properties examined the relationship between metabolite structure and its antioxidative potential (Born et al., 1996). Mesuaxanthone A, isolated from the roots of *Chironiu krehsii* (Gentianaceae), exerted low oxidation potential measured by cyclic voltammetry. Although low oxidation potential and strong antioxidant properties are usually in good correlation, mesuaxanthone A showed obvious, but extremely modest antioxidant properties against ROS-induced lipid peroxidation of brain synaptosomes ($IC_{50} > 100\ \mu M$) and 2,2-azobis (2-amidinopropane) (AAPH)-triggered human serum albumin oxidation ($IC_{50} > 100\ \mu M$). The authors speculated that some other characteristic of the xanthone moiety, apart from oxidation potential, was involved in mesuaxanthone A antioxidant potential. The more recent study confirmed the modest antioxidative activity of mesuaxanthone A. Namely, xanthone inhibited butyrylcholinesterase oxidation induced by AAPH with $IC_{50} = 350 \pm 40\ \mu M$ (Salvi et al., 2002), while IC_{50} values of the standard antioxidants were less than 9 μM.

TABLE 19.16 Endangered Gentianaceae species containing eustomin and demethyleustomin.

Eustomin (1-hydroxy-3,5,6,7,8-pentamethoxyxanthone)	Plant species	References
	*Centaurium erythraea*	Aberham et al. (2011)
	Centaurium erythraea subsp. *erythraea*[a]	Jankovic et al. (2000)
Demethyleustomin (1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone)	**Plant species**	**References**
	*Centaurium erythraea*	Aberham et al. (2011)
	Centaurium erythraea subsp. *erythraea*[a]	Jankovic et al. (2000)

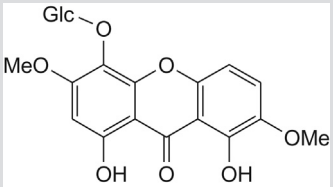
[a]*Protected in the Republic of Serbia.*

TABLE 19.17 Endangered Gentianaceae species containing corymbiferin.

Corymbiferin (1,3,8-trihydroxy-4,5-dimethoxyxanthone)	Plant species	References
	*Gentianella albanica*[a]	Janković et al. (2005), Krstić-Milošević et al. (2015)

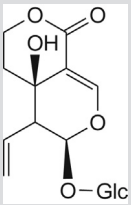
[a]*Protected in the Republic of Serbia.*

TABLE 19.18 Endangered Gentianaceae species containing lanceoside.

Lanceoside (1,8-dihydroxy-3,7-dimethoxyxanthone-4-*O*-β-D-glucoside)	Plant species	References
	*Gentianella albanica*[a]	Janković et al. (2005), Krstić-Milošević et al. (2015)

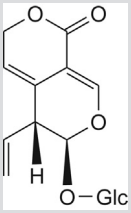
[a]*Protected in the Republic of Serbia.*

TABLE 19.19 Endangered Gentianaceae species containing swertiamarin.

Swertiamarin (swertiamarine, swertiamaroside)	Plant species	References
	*Gentiana asclepiadea*[a]	Šavikin et al. (2015)
	Gentiana cruciata[a]	Mihailović et al. (2015)
	Gentiana dinarica[a]	Krstić et al. (2004), Šavikin et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)
	Gentiana lutea[a]	Šavikin et al. (2015)
	Gentiana pneumonanthe[a]	Popović et al. (2019)
	Gentiana punctata[a]	Menkovic, Savikin-Fodulovic, Vinterhalter, Vinterhalter, and Grubisic (1998), Šavikin et al. (2015)
	Gentianella albanica[a]	Krstić-Milošević et al. (2015)
	Centaurium erythraea[a]	Šiler et al. (2012)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)
	Gentiana kuroo	Wani et al. (2013)
	Centaurium erythraea	van der Sluis (1985)
	Centaurium pulchellum	van der Sluis (1985), van der Sluis and Labadie (1981)
	Centurium somedanum	van der Sluis (1985)
	Swertia longifolia	Saeidnia, Ara, Hajimehdipoor, Read, Arshadi, & Nikan (2016)

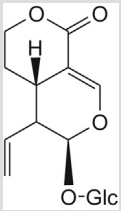
[a]*Protected in the Republic of Serbia.*

TABLE 19.20 Endangered Gentianaceae species containing gentiopicroside.

Gentiopicroside (gentiopicrin)	Plant species	References
	*Gentiana asclepiadea*[a]	Šavikin et al. (2015)
	Gentiana cruciata[a]	Mihailović et al. (2015)
	Gentiana dinarica[a]	Krstić et al. (2004), Šavikin et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)
	Gentiana lutea[a]	Šavikin et al. (2015)
	Gentiana pneumonanthe[a]	Popović , Krstić-Milošević, Stefanović, Matić, Vidaković, and Bojović (2019)
	Gentiana punctata[a]	Menkovic et al. (1998), Šavikin et al. (2015)
	Gentianella albanica[a]	Krstić-Milošević et al. (2015)
	Centaurium erythraea[a]	Šiler et al. (2012)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)
	Gentiana kurroo	Wani et al. (2013)
	Gentiana purpurea	Suhr et al. (1978)
	Gentianella rupicola	Vidari and VitaFinzi (2010)
	Centurium erythraea	van der Sluis (1985)
	Centaurium pulchellum	van der Sluis (1985), van der Sluis and Labadie (1981)
	Centurium somedanum	van der Sluis (1985)

[a]*Protected in the Republic of Serbia.*

TABLE 19.21 Endangered Gentianaceae species containing sweroside.

Sweroside	Plant species	References
	*Gentiana asclepiadea*[a]	Šavikin et al. (2015)
	Gentiana cruciata[a]	Mihailović et al. (2015)
	Gentiana dinarica[a]	Krstić et al. (2004), Šavikin et al. (2015)
	Gentiana kochiana[a]	Šavikin et al. (2015)
	Gentiana lutea[a]	Šavikin et al. (2015)
	Gentiana pneumonanthe[a]	Popović et al. (2019)
	Gentiana punctata[a]	Menkovic et al. (1998), Šavikin et al. (2015)
	Centaurium erythraea[a]	Šiler et al. (2012)
	Swertia punctata[a]	Menković et al. (2002), Šavikin et al. (2015)
	Gentiana kurroo	Wani et al. (2013)
	Centaurium erythraea	Sakina and Aota (1976)
	Centaurium pulchellum	van der Sluis (1985), van der Sluis and Labadie (1981)

[a]*Protected in the Republic of Serbia.*

In a study of Chen, Chen, and Duh (2004), mesuaxanthone A along with three new, and various known compounds were isolated from the roots of *Garcinia linii* (Gutifferae), a small endemic evergreen tree. The cytotoxic potential of isolated metabolites was determined in vitro against mouse lymphocytic leukemia cell line P-388, and the human colon carcinoma cell line HT-29. Xanthone of interest, mesuaxanthone A, exhibited EC_{50} value of 2.76 μg/mL toward the P-388 cell line, but only marginal cytotoxicity against HT-29 cells ($EC_{50} = 7.51$ μg/mL). The antimicrobial activity of mesuaxanthone A and its structural analogue 1,7-dihydroxy-3-methoxyxanthone, isolated from the root of *G. linii* were then investigated (Chen et al., 2004). The anti-tubercular activity of 1,7-dihydroxy-3-methoxyxanthone and mesuaxanthone A was evaluated using the strain of *Mycobacterium tuberculosis* 90-221387 in vitro. Both investigated secondary metabolites showed anti-tubercular activities with MICs (minimal inhibitory concentrations) of 3.1 ± 0.5 and 6.3 ± 1.0 μg/mL, respectively. The MIC values were comparable to clinically used anti-tubercular agent ethambutol. The results indicated that the 3-methoxy group had a significant role in the anti-tubercular potential of mesuaxanthone A.

In addition, the anti-inflammatory potential of mesuaxanthone A isolated from roots of plant *Garcinia subelliptica* (Guttiferae) was explored (Chen et al., 2017). Four xanthones were investigated for anti-inflammatory capacity: mesuaxanthone A, subelliptenone E, garcisubellone, and 1,4,5-trihydroxyxanthone. The anti-inflammatory properties were ascribed only to garcisubellone and 1,4,5-trihydroxyxanthone, since they expressed the potential to suppress formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced superoxide ($O_2^{\cdot -}$) accumulation in human neutrophils.

19.5.3 Gentisein

Gentisein is a xanthone with a simple oxygenation pattern and the most primitive biosynthetic pathway. It serves as a chemotaxonomic marker for five Gentianaceae genera—*Anthocleista*, *Blackstonia*, *Gentianopsis*, *Macrocarpaea*, and *Orphium* (Jensen & Schripsema, 2002). Although present in some *Gentiana* and *Swertia* species, gentisein is not predominant in these genera. The vast majority of gentisein pharmacological activities presented here were gained using plant material from families other than Gentianaceae.

Teng, Lin, Ko, Cheng, and Huang (1989) tested 10 xanthones and their glycosides from the aerial parts of *Tripterospermum taiwanense* and *Tripterospermum lanceolatum* (Gentianaceae) for antiplatelet activity in the collagen-induced aggregation of rabbit platelets. Gentisein, norathyriol (1,3,6,7-tetrahydroxy xanthone), and lancerin acetate (4-*C*-glucosyl-1,3,7-trihydroxyxanthone acetate) showed effective antiplatelet potential at concentrations higher than 50 μg/mL. Tripteroside acetate (6-*O*-β-D-glucosyl-norathyriol acetate) and norathyriol acetate were the most potent inhibitors with minimal effective concentrations around 10 μg/mL. The mechanism of antiplatelet action was further

evaluated using tripteroside acetate and results have shown that the xanthone can profoundly inhibit thromboxane B2 formation caused by arachidonic acid, collagen, thrombin, and ionophore A23187. In addition, it attenuated phosphoinositide breakdown triggered by thrombin, collagen, and platelet-activating factor (PAF), thus suppressing two important initial steps in platelet activation. The authors have concluded that xanthones with three hydroxyl groups (1,3,7-trihydroxyxanthone) were robust antiplatelet agents. Glycosylation decreased the antiplatelet activity, presumably due to increased hydrophilicity of the molecule, which probably reduced penetration into target platelets. Alternatively, acetylation of xanthones or xanthone glycosides significantly enhanced the inhibitory potential toward platelet aggregation. Overall, the modification of xanthone scaffold could lead to the development of more potent antiplatelet agents.

Tzankova, Nedialkov, Kitanov, and Danchev (2010) explored the influence of gentisein and two other constituents (phloroglucinol hyperatomarin and benzophenone annulatophenone) of *Hypericum annulatum* (Clusiaceae) on serotonin binding to $5HT_{1B}$ receptors and serotonin uptake in rat brain sinaptosomes in vitro. All tested naturally derived molecules inhibited serotonin uptake in micromolar concentrations, with gentisein showing the most potent activity ($IC_{50} = 4.7\ \mu M$). In contrast, the investigated xanthones had no ability to bind the $5HT_{1B}$ receptors in rat striatum and hippocampus. In conclusion, the authors associated the well-documented anti-depressant action of *Hypericum* extract with its ability to inhibit serotonin uptake, rather than with a direct effect of *Hypericum* constituents on 5-HT_{1B} binding sites.

Anti-proliferative activity of gentisein and 28 natural and nonnatural hydroxylated and prenylated xanthones was investigated against five human cancer cell lines—HepG2 (hepatocelluar carcinoma), HCT-116 (colon carcinoma), A549 (lung carcinoma), BGC823 (gastric carcinoma), and MDAMB-231 (breast carcinoma) in vitro (Zhang et al., 2012). An MTT test showed that xanthones with prenyl substituents possessed potent anti-proliferative activities against the majority of tested cancer cell lines with IC_{50} values $\leq 10\ \mu M$. Gentisein and several hydroxylated xanthones exerted moderate cytotoxicity with IC_{50} values $\leq 20\ \mu M$ against BGC823, HepG2, and HCT-116 cell lines. The xanthone core prenylation remarkably enhanced its anti-tumor potential. Apart from the introduction of prenyl moiety, the position and number of hydroxyl groups significantly influenced anti-tumor capacity. Thus the hydroxyl group at the C1 or C8 ortho to the carbonyl function in xanthone scaffold seems to be necessary for inhibitory activity toward tumors. The enhancement of binding affinity to the potential biomolecular targets could be attributed to the abovementioned moieties that could serve as hydrogen bond donors and acceptors. Indeed, the introduction of one OH group in compounds with 1,3-dihydroxy substitution improved anti-tumor activity and, gentisein showed supreme potential against HepG2 and A549 cells compared with 1,3-dihydroxy xanthone. Another interesting observation came from this study — most of the investigated compounds showed selectivity toward HepG2 (hepatocellular), HCT-116 (colon), and A549 (lung) cell lines, with moderate or no activity against MDAMB-231, the human estrogen receptor-negative breast carcinoma cell line. Due to the comprehensive insight into the correlation between structures and anti-tumor activities, this work provided important information that could lead to the design of xanthone derivatives with inhibitory potential toward tumors.

In a similar study, cytotoxic activities of gentisein and five natural benzophenones isolated from the aerial parts of *H. annulatum* (Clusiaceae) were assessed in HL-60 (acute promyelocyte leukemia), K-562 (chronic myeloid leukemia), and multidrug resistance-variant of HL-60/Dox (selected in doxorubicin-containing medium) cells (Biljali et al., 2013). This research was aimed to evaluate the ability of investigated compounds to increase chemosensitivity of resistant HL-60/Dox cells to the anthracyclines doxorubicin, eprirubicin, idarubicin, and daunorubicin. Gentisein exerted the highest cytotoxic potential in all tested cancer cell lines. Furthermore, gentisein, in nontoxic concentration, most potently increased chemosensitivity of HL60/Dox cells to anthracyclines. The observed potential of gentisein was probably a consequence of its lipophilicity, which enabled it to interact with an ATP-binding domain or other intracellular epitopes on the surface of the drug transporter responsible for multidrug resistance. The results of this study indicated that the tested xanthone and polyphenols are attractive candidates for future more exhaustive evaluation as drug resistance reverting agents.

Antioxidant activity of gentisein isolated from the stem bark of *Garcinia atroviridis* (Clusiaceae) was evaluated using DPPH radical scavenging test (Tan, Khairuddean, Wong, Tong, & Ibrahim, 2016). Gentisein showed significant antioxidant activity ($EC_{50} = 16.20\ \mu g/mL$), which was comparable to standard antioxidant ascorbic acid ($EC_{50} = 7.4\ \mu g/mL$). Conversely, a study by Meechai, Phupong, Chunglok, and Meepowpan (2016) undertaken to evaluate the antiradical activity of xanthones and flavonoids isolated from the branch of *Garcinia schomburgkiana* (Clusiaceae) showed no significant antioxidant action of gentisein.

An interesting pharmacological activity of gentisein, based on the traditional use of *Polygala tenuifolia* (Polygalaceae) roots for the treatment of psychiatric disorders in ancient China, was investigated by Yang, Xu, Zhao, Gu, and Wang (2018a). Explicitly, a modulatory potential of gentisein on neurotrophic factors synthesis in rat astrocyte

primary cultures was evaluated. Gentisein significantly stimulated mRNA expression and enhanced protein levels of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in cultured rat astrocytes. The underlying mechanism of gentisein neurotrophic activity implicated upregulation of enzymes involved in the synthesis of NGF and BNDF, in particular the tissue plasminogen activator/plasmin system. Protein kinase A and extracellular signal-regulated kinases (ERK) inhibitors significantly inhibited gentisein-induced NGF and BDNF expressions in rat astrocytes, implying pivotal roles for both cyclic adenosine monophosphate (cAMP)- and ERK-dependent pathways in the observed effect. These results support the potential use of gentisein in treating neurodegenerative disorders.

19.5.4 Gentisin and isogentisin

Gentisin from *G. lutea* has been associated with the potential to inhibit the aberrant proliferation of vascular smooth muscle cells (VSMC), a typical feature of restenosis (Waltenberger et al., 2015). Using the resazurin conversion method, Waltenberger et al. (2015) found that gentisin suppressed platelet-derived growth factor (PDGF)-induced muscle cell proliferation ($IC_{50} = 7.84\ \mu M$). The anti-proliferative capacity of gentisin was additionally confirmed using 5-bromo-2′-deoxyuridine (BrdU) incorporation assay ($IC_{50} = 5.74\ \mu M$). Three other xanthones — swerchirin, 1-hydroxy-2,3,4,5-tetramethoxyxanthone, and methylswertianin from *Frasera caroliniensis* and *C. erythraea*, showed the similar potential to interfere with VSMC proliferation as gentisin. The potential of investigated xanthones to interfere with VSMC proliferation represents a promising starting point for development of drugs that could prevent pathological re-narrowing of the blood vessel lumen after surgical treatment of stenosis.

Further, there are reports on the antioxidant, antimicrobial, and anti-cancer in vitro activities of the *Gentiana kurroo* and *Gentiana macrophylla* extracts containing gentisin (Wani et al., 2013; Yin, Xie, & Guo, 2018), but the exact contribution of gentisin to those effects was not evaluated in detail.

Isogentisin, an isomer of gentisin, and its 3-*O*-glucoside were among the first xanthones demonstrated to possess monoamine oxidase A (MAO A) and monoamine oxidase B (MAO B) inhibitory activity (Suzuki et al., 1981). Although this property underlined them as possible anti-depressant agents, in vivo ability of these xanthones has not been assessed yet. However, convincing evidence exists about the vasculoprotective and gastroprotective properties of isogentisin. Namely, in a search for pharmacologically active compounds from 22 alpine plant extracts, Schmieder et al. (2007) found that the methanol extract of Gentian root and its constituent isogentisin were the most effective in the protection of human vascular endothelial cells against cigarette smoke extract (CSE)-induced injury. Time-course analysis has shown that isogentisin probably activated cellular machinery responsible for degradation of oxidatively modified proteins, at least partly stabilized the microtubule system, and subsequently exerted cytoprotective action. In a similar manner, by investigating potentially active compounds from Brazilian medicinal plants, Klein et al. (2010) reported the pronounced gastroprotective activity of acetone extract and methanol fractions of *Polygala cyparissias* (Polygalaceae), and the main constituents of acetone extract isogentisin, 1,7-dihydroxy-2,3-methylenedioxyxanthone, and alpha-spinasterol. Namely, acute administration of isolated xanthones and sterol at a dose of 50 mg/kg (p.o.) to mice significantly reduced the percentage of the lesion, total lesion area, and ulcerative lesion index in the ethanol/HCl-triggered ulcer model. Although underlying mechanisms were not evaluated in this study, the authors speculated that gastroprotective potential could be partly explained by the presence of both sterol and xanthones, since their effect overcame the activity of methanol fractions in vivo.

However, the fact that gentisin and isogentisin were identified as responsible for the mutagenic activity of Gentian root methanol extract in Ames test needs special attention (Morimoto et al., 1983). Based on this report, Gentian root was classified as a potential mutagen in pregnancy and lactation (Mills, Duguoa, Perri, & Koren, 2006).

19.5.5 Demethylbellidifolin

Demethylbellidifolin (DMB) is simple, tetraoxygenated xanthone, most widely observed in *Swertia* species (Li, Zhao, Huang, & Wang, 2017).

DMB, isolated from the whole plant *Gentianella acuta*, showed cardioprotective effects against myocardial cell injury induced by oxidative stress (Wang et al., 2018). DMB in a dose-dependent manner (concentrations ranging from 50 to 200 μM) increased the viability of rat embryonic H9C2 cardiomyocytes treated with hydrogen peroxide (H_2O_2). This finding was in concordance with the ability of DMB (in dose 100 or 300 μg/mL) to significantly recover the cardiac function during reperfusion in isolated rat hearts (Jiang et al., 2002). Similarly, DMB, isolated from *G. acuta*, showed strong dose-dependent protective activity against H_2O_2-induced cell death of PC12 rat pheochromocytoma (Lv, Ren, Na, & Li, 2017). The strongest effect DMB displayed at a concentration of 50 μM. Further, DMB, isolated from

Swertia mussotii, exerted antioxidative capacity against H_2O_2-triggered death of pancreatic β-cell line (INS-1) (Zheng et al., 2014a, 2014b). It should be pointed out that in this model DMB (concentration of 0.3 and 5 μm) showed better antioxidative properties than quercetin, a well-known antioxidant (Zheng et al., 2014a, 2014b).

Having in mind that oxidative stress is considered to be the trigger for the development of type 2 diabetes and that DMB possesses antioxidative capacity, anti-diabetic potential of DMB was investigated. Zheng et al. (2014a, 2014b) reported the ability of DMB to inhibit two enzymes upregulated in diabetes type 2, aldose reductase and α-glucosidase ($IC_{50} = 5.2 \pm 0.3$ μM and $IC_{50} = 88.6 \pm 1.6$ nM, respectively). Antioxidative capacity together with the potential to modulate the target molecules in diabetes type 2 makes DMB a possible novel multitarget-directed anti-diabetic agent that could be used for the treatment of diabetes and diabetes-related complications.

The antioxidative activity of DMB was shown using the 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide radical (PTIO) trapping model. Li et al. (Li, Chen, Zhao, & Chen, 2018) observed that DMB in a dose-dependent manner scavenged the PTIO radical at pH 4.5 and 7.4. Further investigation of 16 different natural xanthones led to the conclusion that the position of the OH group in the phenolic ring significantly affected antioxidant ability (Li et al., 2018). Namely, xanthones with two OH groups in the *para* or *ortho* position exhibited lower IC_{50} values compared to other investigated compounds. Four substituent types (i.e., *para*-di-OHs, 5,6-di-OHs, 6,7-di-OHs, and 7,8-di-OHs) were responsible for the antioxidant activity of phenolic xanthones, while other substituents (like isoprenyl and 3-hydroxy-3-methylbutyl) had only a slight effect (Li et al., 2018). In concordance with the latter, strong DPPH free radical scavenging effects, as well as ferric-reducing antioxidant power (FRAP), of the extracts isolated from aerial parts of *Gentianella multicaulis*, was ascribed to extracts active components—DMB and bellidifolin (Lima et al., 2012). Also, the extract, DMB, and bellidifolin showed a modest antifungal effect against the dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* (Lima et al., 2012).

One of the widely accepted approaches for the treatment of Alzheimer's disease, a devastating chronic neurodegenerative disorder, is to increase the level of acetylcholine (ACh) by inhibiting acetylcholinesterase (AChE) enzyme. Urbain, Marston, Queiroz, Ndjoko, and Hostettmann (2004) carried out an extensive study investigating more than 50 plant methanol extracts to identify ones with the potential to inhibit AChE. They detected the methanol extract of *Gentiana campestris* leaves to contain several molecules with anti-AchE activity, among which DMB was identified. Unlike Kang and Fang (1997), who speculated that oxygenated polycyclic aromatic hydrocarbons, such as xanthones, should have little effect on AchE activity, Urbain et al. (2004) clearly demonstrated that this class of compounds represents respectable AchE inhibitors. Namely, bellidifolin, a structural analog of DMB, exhibited the most active property; it inhibited the target enzyme almost as identically as galanthamine, the commercial AchE inhibitor, with a minimum inhibitory quantity of 0.01 μg. Similarly, DMB revealed an excellent minimum inhibitory quantity of 0.04 μg (0.15 nM) giving it a place in further studies.

Besides previously showed numerous DMB bioactivities, it was found that DMB possesses a positive role in atherosclerosis prevention, too. The main characteristic of this progressive, inflammatory disease is the formation of atherosclerotic plaques, which consist of lipids, endothelial, and immune cells (Woollard & Geissmann, 2010). Jiang et al. (2003) showed that DMB, isolated from *Swertia davidi*, attenuated the adhesion of monocytes to endothelial cells induced by oxidized low-density lipoprotein (ox-LDL) in vitro. The observed protective effect of DMB is probably due to a reduction in the activity of asymmetric dimethylarginine (ADMA), endogenous nitric oxide synthase (eNOS) inhibitor, through tumor necrosis α (TNF-α) downregulation. Since atherosclerosis is associated with the reduced nitric oxide (NO) levels (Kawashima & Yokoyama, 2004), blocking endogenous NO inhibitor with DMB could potentially benefit the bioavailability of NO and subsequently exert a positive role on the impairment of endothelium-dependent relaxations, the distinctive feature of atherosclerosis. Similarly, the potential of DMB, extracted from *S. davidi*, to decrease ADMA concentration, through a boost in the activity of the enzyme important in ADMA degradation (dimethylarginine dimethylaminohydrolase), showed a protective effect toward endothelium cells treated with lysophosphatidycholine in vitro and ox-LDL in vivo (Jiang et al., 2004).

Nitroglycerin (NTG), an important cardiovascular drug, used against cardiac disorders like hypertension, angina pectoris, and congestive heart failure, is converted by vascular aldehyde dehydrogenase (ALDH-2) to NO, which subsequently activates a signaling cascade leading to vasodilation. Despite that, long-term treatment with nitroglycerin could cause drug tolerance. Since there are substantial data on the relationship between nitroglycerin tolerance development and increased oxidative stress, the potential role of DMB as an antioxidant in this process was investigated in vitro and in vivo (Shi et al., 2009). While a single dose of nitroglycerine significantly decreased blood pressure in rats, 8-day-long pretreatment with nitroglycerin almost completely reduced the depressor effect of nitroglycerin. Similarly, NTG considerably downregulated the tension of isolated aortic rings induced by phenylephrine in vitro, whereas 30 min treatment of the ring segments with a high concentration of NTG reduced their sensitivity to subsequent relaxation via

NTG. Pretreatment with DMB, a major xanthone compound of *S. davidi*, completely abolished drug tolerance development, both in vivo (10, 30, 90 mg/kg once a day orally for 9 days) and in vitro (3, 10, and 30 μM). In addition, pretreatment of cultured human umbilical vein endothelial cells (HUVECs) with DMB, prevented increased ROS production, attenuated cyclic guanosine monophosphate (cGMP) levels, and decreased the activity of ALDH-2 induced by incubation of cells with NTG. In summary, DMB could prevent NTG tolerance, probably due to antioxidant properties and the ability to restore ALDH-2 activity.

Among extensive pharmacological actions, DMB showed the anti-fibrotic effect in the culture of hepatic stellate cells (HSC) (Li, Lu, & Zou, 2011). HSC are the major cell type involved in liver fibrosis, that is, the formation of scar tissue in response to liver damage. Different concentrations of DMB (10, 20, or 40 μM), extracted from *S. davidi*, markedly inhibited proliferation of activated rat HSC-T6 line, and downregulated expressions of both α-smooth muscle actin (SMA) and collagen I, markers of HSC activation. In addition, DMB in a dose-dependent manner downregulated mRNA and protein expression of transforming growth factor-β1 (TGF-β1) and its downstream molecule connective tissue growth factor (CTGF). On the other hand, reduced expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) detected in activated HSC-T6 cells, was significantly enhanced by treatment with DMB, suggesting that antiproliferative effect of DMB may be related to PPAR-γ activation. These results advocated DMB as an effective antifibrotic drug.

19.5.6 Bellidifolin

Although bellidifolin, a simple tetraoxygenated xanthone, is isolated from *Gentiana bellidifolia* in 1964 (Markham, 1964), its pharmacological activities have not been investigated until the 1990s. In the first reported pharmacological study hypoglycemic potential of bellidifolin was evaluated (Basnet, Kadota, Shimizu, & Namba, 1994). Five xanthones and two triterpenoids were isolated from the ethyl acetate extract of *Swertia japonica* whole plant. Among them, bellidifolin and methylbellidifolin (1,8-Dihydroxy-3,5-dimethoxy xanthone; swerchirin) showed the most potent activity in lowering the blood glucose level in streptozotocin (STZ)-treated rats. Bellidifolin reduced blood glucose levels by 28.9% after subchronic (5 doses, 50 mg/kg each) i.p. administration, and by 20.9% 3 h after single p.o. administration (50 mg/kg). Furthermore, bellidifolin lowered triglyceride (TG) levels in normal and STZ-treated rats and stimulated 2-deoxy-glucose uptake in HIRcB fibroblast cells with a potency similar to insulin (Basnet et al., 1995). More detailed analysis of hypoglycemic and hypolipidemic effects of bellidifolin and methylswertianin from the ethyl acetate fraction of *Swertia punicea* in STZ-induced type 2 diabetic mice was conducted by Tian et al. (Tian et al., 2010). The xanthones were administered to the mice orally (200 and 100 mg/kg body wt/day) for 4 weeks. Both compounds significantly reduced fasting blood glucose, improved oral glucose tolerance, and lowered fasting serum insulin. Analysis of insulin signaling cascade showed that xanthones can restore STZ-induced decrease in expression levels of insulin-receptor α subunit (InsR-α), insulin-receptor substrate-1 (IRS-1), and phosphatidylinositol 3-kinase (PI3K). These results implied that enhanced insulin signaling triggered by xanthone improved insulin resistance in xanthone-treated animals. Additional mechanisms underlying hypoglycemic action included decreased activity of glucose-6-phosphatase, the enzyme that completes the final step in gluconeogenesis (formation of free glucose), and increased activity of glucokinase, which catalyzes the first step of glycogen synthesis. Through modulation of these enzymes, xanthones increased hepatic glycogen content and regulated hepatic glucose output. The authors have concluded that bellidifolin and methylswertianin could be useful candidates in the therapy of type 2 diabetes, likely via the improvement of insulin resistance.

Traditional use of *S. punicea* in Chinese folk medicine for the treatment of acute and chronic liver diseases has prompted further investigation of its capacity to protect hepatic cells in culture and in vivo (Zheng et al., 2014a, 2014b). The plant active constituents, bellidifolin and 1,7-dihydroxy-3,4,8-trimethoxyxanthone, were evaluated in carbon tetrachloride (CCl_4)- and N-acetyl-p-aminophenol (APAP)-induced HepG2 cell damage. Both xanthones effectively improved CCl_4-induced alteration in levels of aspartate transaminase (AST), lactate dehydrogenase (LDH), SOD, and malonyl dialdehyde (MDA). Furthermore, bellidifolin alleviated APAP-induced hepatotoxicity by increasing glutathione (GSH) content and decreasing ROS production. The 1,7-dihydroxy-3,4,8-trimethoxyxanthone was examined in a model of dimethyl nitrosamine-induced rat hepatic fibrosis in vivo and improved all measured serum markers (albumin, alanine aminotransferase, AST, total bilirubin, SOD, and MDA) and histopathological features of hepatic injury. Overall, bellidifolin and 1,7-dihydroxy-3,4,8-trimethoxyxanthone can serve as candidates for the development of hepatoprotective agents.

The ability of bellidifolin to enhance antioxidative stress defense and thus manifest hepatoprotective capacity is not surprising, considering numerous reports about radical scavenging activities of xanthone derivatives (Blanco-Ayala et al., 2013; Jung, Su, Keller, Mehta, & Kinghorn, 2006; Mahendran, Manoj, Rajendra Prasad, & Narmatha Bai, 2015).

Accordingly, in the evaluation of radical-scavenging properties of seven active components from *S. chirayita* using DPPH test, bellidifolin exerted significant DPPH neutralizing effect ($IC_{50} = 17.45$ μg/mL) (Singh, Ambika, & Chauhan, 2012). Structure−activity relationship analysis revealed the importance of the *para*-quinonoid structure, as well as the electron-donating property of the *ortho*-alkoxy group for the antioxidant activity of bellidifolin.

Furthermore, bellidifolin has shown neuroprotective action in a hypoxia-induced injury of PC12 pheochromocytoma cells (Zhao et al., 2017). The protection was based on the ability of bellidifolin to inhibit hypoxia-induced activation of proapoptotic p38 mitogen-activated protein kinase (MAPK) signaling pathway and subsequently reduce the expression level of apoptosis executive caspase-3.

The most recent study has evaluated anti-inflammatory properties of bellidifolin and swerchirin isolated from petrol ether and ethyl acetate fractions of *S. chirayita* (Hu et al., 2018). Both xanthones inhibited production of the proinflammatory cytokines interleukin-6 (IL-6) and TNF-α in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Additionally, bellidifolin suppressed expression of cyclooxygenase-2 (COX-2) and subsequently reduced the production of inflammatory mediator prostaglandin E2 (PGE2). Anti-inflammatory action of bellidifolin was mediated through a reduction in LPS-stimulated activation of c-Jun N-terminal kinases (JNK), ERK, and p38 MAPKs. Also, bellidifolin significantly repressed the activity of the main proinflammatory transcription factor nuclear factor-kappa B (NF-κB), by inhibiting activities of its upstream activators IKKβ and Akt. The activity of bellidifolin in LPS-stimulated macrophages suggests that it is a good therapeutic candidate for the management of inflammatory-mediated immune disorders.

Bellidifolin and three other xanthones (demethylbellidifolin, bellidifolin 8-*O*-β-glucopyranoside (swertianolin), bellidin-8-*O*-β-glucopyranoside (norswertianolin)) were isolated from a methanol extract of *G. campestris* leaves and found to inhibit acetylcholinesterase (AChE) activity using thin-layer chromatography (TLC)-bioautography (Urbain et al., 2004). Bellidifolin was the most potent compound, with activity similar to standard AChE inhibitor galanthamine. Both aglycones (bellidifolin, bellidin) were more active than corresponding glycosides, probably due to hydrophobicity or steric factors. Although xanthones have shown significant potential to inhibit AchE, the authors have emphasized the necessity of more comprehensive testing before considering them as novel drugs for Alzheimer's disease.

In a similar study, bellidifolin and nine other xanthones were isolated from the whole Mongolian plant *Gentianella amarella* and tested in vitro using TLC-bioautography for MAO and AChE inhibiting activity (Urbain et al., 2008). Bellidifolin and DMB showed potent MAO A inhibitory activity, causing 90.5% and 98.9% enzyme inhibition at 10 μM, respectively. Swertianolin, the 8-*O*-glucopyranoside form of bellidifolin, inhibited 93.6% of MAO B activity at 10 μM concentration. These findings underlined xanthones as potential lead compounds in developing new drugs for aging-related neurodegenerative diseases (Cruz, Cidade, & Pinto, 2017). A similar level of MAO A inhibition by bellidifolin and DMB was displayed using radioligand binding assays (Tovilovic, Tomic, Jankovic, & Krstic, 2005). Bellidifolin, DMB, and corymbiferin, from diethyl ether extract of *Gentianella austriaca*, inhibited MAO A with IC_{50} values of 3.40 μg/mL (extract), 1.10 μM (bellidifolin) and 2.14 μM (DMB). The ability to inhibit MAO B isoform was less pronounced ($IC_{50} = 260$ μg/mL, 2490 μM, and 368 μM for extract, bellidifolin, and DMB, respectively). Corymbiferin did not show significant MAO A or B inhibitory effect. Although marked MAO A blocking ability of the extract and xanthones implied a possibility to induce behavioral modulation, additional in vivo studies are necessary to provide a reliable estimation of their anti-depressant potential.

19.5.7 Swerchirin

Although swerchirin is present in shoots and roots of the plant *Centaurium pulchellum*, which is protected in the Republic of Serbia (Krstić, Janković, Šavikin-Fodulović, Menković, & Grubiŝić, 2003), it is most widely observed in *Swertia* species (Li et al., 2017).

As previously mentioned, swerchirin and bellidifolin isolated from the whole plant of *S. chirayita* commonly used in traditional Tibetan medicine, showed significant anti-inflammatory properties in LPS-stimulated RAW 264.7 murine macrophages (Hu et al., 2018). Both isolated xanthones, separately, downregulated the LPS-stimulated production of the proinflammatory cytokines IL-6 and TNF-α in vitro. In addition, swerchirin in a dose-dependent manner restrained the production of PGE2, the important inflammatory mediator, in activated macrophages (Hu et al., 2018), indicating swerchirin and bellidifolin as potential biotherapeutics for inflammatory-mediated diseases.

Swerchirin in a dose-dependent manner exhibited an anti-proliferative effect against human ovarian cancer SKOV3 cells in vitro ($IC_{50} = 20$ μM at 48 h) (Luo, Zhao, & Luo, 2018). The cell cycle distribution by flow cytometry demonstrated that swerchirin induced G_2 phase cell cycle arrest in a dose-dependent manner (10−40 μM). Similarly, concentration-dependent disruption of mitochondrial membrane potential, followed by a significant increase in annexin V/propidium iodide positive (annexin V^+/PI^+) swerchirin-treated cells (from 5.2% in control to 67.8% at 40 μM

concentration) was noticed. Furthermore, treatment with swerchirin led to the decrease in expression of anti-apoptotic protein Bcl-2 and enhanced expression of proapoptotic molecules, cytosolic cytochrome c, Bax, cleaved caspase-3, and cleaved caspase-9, compared to the untreated cells. Interestingly, swerchirin induced anti-cancer action partially through the Raf/MEK/ERK signaling pathway, since in its presence protein expressions of phospho-MEK and phospho-ERK were decreased.

As mentioned above (Section 19.1.4) swerchirin, like some other xanthones (gentisin, 1-hydroxy-2,3,4,5-tetramethoxyxanthone, and methylswertianin), possessed the ability to interfere with the aberrant proliferation of VSMC (Waltenberger et al., 2015). Swerchirin from the methanol extract roots of *F. caroliniensis* and *C. erythraea*, attenuated PDGF-triggered proliferation of VSMC cells, with an IC_{50} value of 12.5 μM. For comparison, taxol, the well-known anti-proliferative drug, exerted an inhibitory effect with an IC_{50} value of 0.1 μM. To confirm the results obtained with the resazurin conversion method, PDGF-stimulated VSMC proliferation was measured by BrdU incorporation assay. Swerchirin in a dose-dependent manner constrained VSMC proliferation provoked with PDGF ($IC_{50} = 7.37$ μM). Concurrently, no evidence of swerchirin cytotoxic potential was reported.

Bajpai et al. (Bajpai, Asthana, Sharma, Chatterjee, & Mukherjee, 1991) identified swerchirin, isolated from the hexane fraction of the *S. chirayita*, as a compound with the capacity to significantly lower blood glucose level in fasted, fed, glucose loaded, and tolbutamide pretreated albino rat models. Swerchirin showed a hypoglycemic effect ($ED_{50} = 23.1$ mg/kg/oral) which is most probably consequence of swerchirin-triggered insulin release from Langerhans islets (Saxena, Bajpai, Murthy, & Mukherjee, 1993). Similarly, swerchirin (hexane fraction of *S. chirayita*) showed the ability to lower blood sugar level in both healthy and STZ-treated rats (Saxena, Murthy, & Mukherjee, 1996). Detailed analysis of the crude aqueous and ethanol extracts of *S. chirayita* collected from nine districts of Nepal showed a moderate-to-high positive correlation of antioxidant and α-glucosidase inhibitory activity with total phenolic content of both extracts (Phoboo et al., 2013). Interestingly, although in previous studies the anti-diabetic property of *S. chirayita* is attributed to swerchirin, the authors propose that the other xanthones also may contribute to the anti-hyperglycemia potential of the crude plant extract (Phoboo et al., 2013).

Furthermore, there is an evidence that swerchirin, from the whole plant of *Swertia calycina*, possesses a protective effect on hematopoiesis in mice irradiated with 550 rad ^{60}Co gamma rays (Ya, Nian, Li, & Gen, 1999). Intraperitoneal acute administration of swerchirin (10 mg/kg) triggered a peripheral blood leukocytes proliferation and an augmentation in serum colony stimulating factor (CSF). In addition, treatment with swerchirin (10 mg/kg, 3 time/week, i.p.) induced a substantial increase of colony formation in the spleen, as well as an improvement in granulocyte-macrophage colony stimulating factor (GM-CSF)-triggered proliferation of bone marrow cells. The authors speculated that potential of swerchirin to induce leukocyte proliferation is due to its effect on CSFs and other hematopoietic growth factors.

Chemoprotective properties of swerchirin toward photosensitized DNA damage, which has an unquestionably significant role in different phototoxic effects (like UV-induced carcinogenesis, photogenotoxicity, and phototoxicity), are documented too (Hirakawa et al., 2005). Four xanthones (bellidifolin, gentiacaulein, norswertianin, and swerchirin) were enrolled in an investigation of their potential chemoprotective properties toward photosensitized DNA damage induced by riboflavin. Swerchirin showed the capacity to diminish the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine, an oxidative product of guanosine (G), by photoexcited riboflavin. Swerchirin and bellidifolin modestly prevented damage induced by photoexcited riboflavin, while the highest preventive action was shown by gentiacaulein and norswertanin. Although various protective mechanisms of xanthones are ascribed to their antioxidation potential, in this paper the authors speculate that this chemoprevention of gentiacaulein and norswertanin is based on the quenching of a photosensitizer.

Moreover, swerchirin isolated from aerial parts of *Swertia longifolia* demonstrated protective capacity against paracetamol-induced hepatotoxicity in Swiss mice (Hajimehdipoor et al., 2006). Namely, acute pretreatment of mice with total plant extract and swerchirin (6−50 mg/kg, orally), significantly reduced the elevation of the enzymes increased by liver damage- AST, ALT, and alkaline phosphatase.

It is particularly worth mentioning a paper from Sarmah (Sarmah, 2012) who gave a positive prediction of swerchirin and gentianine (4-(2-hydroxyethyl)-5-vinylnicotinate g-lactone) interaction with the glucocorticoid receptor. It was shown that the investigated compounds could bind to the same binding pocket in the ligand-binding domain of cytosolic glucocorticoid receptor (GR) as dexamethasone, a well-known inducer of GR. The binding affinities and the output parameters of swerchirin and dexamethasone were in positive correlation, suggesting similar affinity and efficacy of both compounds toward GR. Further, visualization analysis of the models clearly indicated that both compounds could be GR activators. This study proposed swerchirin and gentianine as potential anti-inflammatory agents and gave a scientific explanation for the traditional use of *S. chirayita* in the relief of cough and asthma attack.

19.5.8 Gentiacaulein and gentiakochianin

Gentiacaulein and gentiakochianin are often investigated together since they are both found in *Gentiana kochiana* roots and leaves (Rivaille, Massicot, Guyot, & Plouvier, 1969). For example, the vasodilator capacity of *G. kochiana*, an Italian traditional anti-hypertensive plant, is attributed to these two xanthones (Chericoni et al., 2003). The first study evaluating pharmacological properties of plant crude methanol extract reported its potential to induce the dilatation of the aortic ring in vitro (Uncini Manganelli, Chericoni, & Baragatti, 2000). Continuing this research, gentiacaulein and gentiakochianin were found to display vasorelaxant activity in rat aortic preparation precontracted with norepinephrine, KCl, or caffeine (Chericoni et al., 2003). A mechanism of their action was assumed to be a blockade of intracellular calcium release from sarco/endoplasmic reticulum via inactivation of ryanodine-sensitive Ca^{2+} channels, although this presumption was not experimentally confirmed.

In accordance with MAO inhibiting activity of several other xanthones, diethyl ether extract of *G. kochiana* aerial parts consisting mostly of gentiacaulein (76.1%) and gentiakochianin (14.2%) was evaluated for anti-depressant and anxiolytic activity in rodents (Tomić et al., 2005; Tovilovic, Krstic, Ignjatovic, Janac, & Tomic, 2011). Both extract and gentiacaulein significantly reduced activity of rat microsomal MAO A ($IC_{50} = 0.22$ μg/mL and 0.49 μM, respectively) in vitro. The extract (10-day s.c.; 20 mg/kg) strongly decreased immobility score in a forced swimming test and inhibited ambulation and stereotypy in an open-field test, indicating anti-depressant and sedative effect in mice (Tomić et al., 2005). The anti-depressant effect was attributed to the MAO A inhibition, while the sedative effect implied possible interaction of xanthones with GABA-ergic system (Tomić et al., 2005). However, this presumption has not been confirmed using in vitro radioligand binding studies (Tovilovic et al., 2011). Despite the evident anxiolytic effect of the extract (acute administration, 10 and 20 mg/kg, Wistar rats) in elevated plus maze test, the mechanisms underlying this effect remained unclear (Tovilovic et al., 2011).

These two xanthones also possess substantial antiglioma capacity in vitro (Isakovic et al., 2008). Gentiacaulein displayed anti-proliferative properties by triggering G_0/G_1 cell cycle arrest in C6 rat glioma and U251 human glioma cell lines. However, despite the capacity to activate mitochondrial depolarization, gentiacaulein did not cause any significant apoptotic effect. In contrast, gentiakochianin showed proapoptotic effect by arresting glioma cells in G_2/M phase, reducing mitochondrial membrane potential, increasing ROS production, and subsequently inducing apoptotic cell death. The G_2/M phase arrest was probably based on its microtubule stabilizing effect. This study emphasized genciakochianin as a promising antitumor agent.

Several bioactivities of gentiacaulein were reported. In a search for a new compound with cyclic AMP- and cyclic GMP (cGMP)-phosphodiesterase inhibitory potency, Ruckstuhl and Landry (Ruckstuhl and Landry, 1981) have found that gentiacaulein effectively inhibited bovine lung cGMP phosphodiesterase activity in vitro ($Ki = 8.6$ μM). Due to this property gentiacaulein may have anti-asthmatic potential. As already mentioned, gentiacaulein exerted a chemopreventive action on DNA damage induced by photosensitized riboflavin (Hirakawa et al., 2005). Among the four examined xanthone derivatives (bellidifolin, gentiacaulein, norswertianin, and swerchirin), gentiacaulein and norswertianin most effectively decreased the photoexcited riboflavin-provoked formation of guanosine oxidative product. The prevention of DNA photodamage resulted from quenching of riboflavin triplet excited state by xanthones. Thus xanthone derivatives, especially gentiacaulein and norswertianin, may be used as a basis for the development of new chemopreventive drugs.

19.5.9 Swertiaperenine

Like many other xanthones, metylswertianin possesses powerful antioxidant capacity. Namely, methylswertianin isolated from *Codonopsis ovata* (Campanulaceae) (0.308% ± 0.03% dry weight), exhibited high DPPH scavenging activity (70.3% ± 1.0%), as well as high antioxidant activity, confirmed by ABTS (2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) assay system at 1 μg/mL (Dar et al., 2014).

The antiplasmodial effects of petroleum ether extract from leaves of *Anthocleista vogelii*, a medicinal plant widely used in Nigeria, against residual infection in chloroquine-sensitive *Plasmodium berghei* infected mice were investigated (Alaribe et al., 2012). Intraperitoneal extract application triggered a dose-dependent decline in parasite density. No oral acute toxicity of the extract was observed. Subsequent phytochemical analysis of *A. vogelii* leaf extract yielded two xanthones: decussatin and swertiaperenine. Since decussatin displayed only a weak reduction of parasite density in vivo, it could be speculated that antiplasmodial potential of the extract can be ascribed to the synergistic action of several active principles.

Furthermore, swertiaperenine alongside five other natural compounds (2-phenylethyl-β-D-glucoside, amaroswerin, norswertianin, swertiamarin, and 1,3,8-trihydroxy-5-methoxyxanthone) isolated from the ethanol extract of *S. mussotii*,

showed anti-inflammatory activity (Xiang, Haixia, Zenggen, & Yanduo, 2019). All isolated compounds exhibited potential to reduce LPS-induced production of NO in mouse RAW 264.7 cells. Swertiaperenine was among the less potent xanthones with the IC_{50} value of 26.06 μg/mL.

In previous sections, two more pharmacological activities of swertaiaperenine, anti-diabetic (Tian et al., 2010) and anti-proliferative (Waltenberger et al., 2015), were described in detail (the pharmacologic activity of bellidifolin and pharmacological activity of swerchirin, respectively).

19.5.10 Decussatin

Antimicrobial properties of decussatin and several other xanthones isolated from the stem bark of *A. vogelii* (Gentianaceae) were investigated against *Candida parapsilosis* (Tene et al., 2008). Decussatin was more efficient (MIC = 25 μg/mL) than 1-hydroxy-3,7-dimethoxyxanthone (MIC = 200 μg/mL), which led to the conclusion that higher antifungal activity could be accounted for by the additional methoxy group at position 8. In contrast, Alaribe et al. (Alaribe et al., 2011) found no significant antimicrobial activity of decussatin, isolated from *Ficus congensis* (Moraceae), against numerous bacteria and fungi strains. Decussatin from the Tibetan plant *Gentianopsis paludosa* (Gentianaceae) showed weak antimicrobial potency against the *Mycobacterium smegmatis* and *M. tuberculosis* (MIC = 128 μg/mL) (Yeung, Lau, Chan, Zong, & Che, 2009). Further, antiplasmodial action of decussatin from petroleum ether extract of *A. vogelii* leaves was assessed against residual infection in chloroquine-sensitive *P. berghei*-infected mice (Alaribe et al., 2012). Although the extract reduced parasite density when given i.p. (50, 100, 250 mg/kg, 4 days), decussatin showed no effect in vivo (10 mg/kg, 4 days). However, the petroleum ether extract and decussatin showed strong iron chelating capacity at the concentration of 1 mg/mL (89.53% ± 1.00%, 77.8% ± 0.5%, respectively) compared to EDTA (118.5% ± 2.78%, 1 mg/mL). The chelating ability of deccusatin could be beneficial against *P. berghei*, since the elimination of the excess toxic iron from the blood could improve disease outcome.

Since *A. vogelii* is used in Cameroonian ethnomedicine for the treatment of stomachache, Ateufack, Nguelefack, Wabo, Tane, and Kamanyi (2014) evaluated possible antiulcer properties of the stem bark of this plant and its constituent decussatin. Oral administration of decussatin (1, 2, and 5 mg/kg, single dose) dose-dependently prevented ulcer induced by HCl/ethanol or indomethacin. In both experimental models, decussatin attenuated gastric acid production and enhanced mucus secretion. It can be concluded that by strengthening gastric mucosal defenses decussatin exerts gastroprotective and antisecretory capacity. Contrary to the observed antiulcer activity which could be responsible for stomachache relieving, decussatin showed marked spasmogenic activity (Ateufack, Nguelefack, Mbiantcha, Tane, & Kamanyi, 2007). Decussatin, isolated from the methanol extract of *A. vogelii* stem bark, produced a concentration-dependent increase of spontaneous rat ileal and gastric smooth muscle contractions. Medium containing nifedipine (calcium channel blocker), atropine (anticholinergic agent), and pyrilamine maleate (specific histamine channel inhibitor) abrogated its effect, proposing that decussatin could display spasmogenic activity through calcium channel, muscarinic, and histaminic receptors activation, probably causing intracellular Ca^{2+} mobilization. Thus further experiments may be necessary to provide a reliable estimate of its effects on the gastrointestinal tract.

Decussatin was found to produce hypolipidemic and hypoglycemic effects in male Wistar rats fed with a high fructose diet (Bao, Hu, Zhang, & Wang, 2016). Following a 12-week administration of xanthones (decussatin, 1-hydroxy-3,5,8-trimethoxyxanthone, methyl swertiamarin, and 6,8-dihydroxy-1,2-dimethoxyxanthone; 20 mg/kg, p.o.), isolated from *Lomatogonium rotatum* (Gentianaceae), the epididymal adipose tissues of rats were lighter, as compared with a fructose-fed group. All measured parameters related to the glucose metabolism in the serum were decreased. Moreover, decussatin opposed fructose-triggered increase in TG and cholesterol levels and reduction in high-density lipoproteins (HDL) levels. The authors speculated that observed hypolipidemic/hypoglycemic action of xanthones was due to its potential to modulate AMP-activated protein kinase (AMPK) activity in the liver, and subsequently reduce fatty acid synthesis and fat accumulation. These findings point out that xanthones may be useful in the treatment of fatty liver and obesity-associated diseases in humans.

Apart from the listed activities, decussatin isolated from *G. paludosa* (Gentianaceae) showed substantial cytotoxicity toward the HL-60 human promyelocytic leukemia cell line (Ding et al., 2009). At lower concentrations (12.4−74.4 μM) it exhibited anti-proliferative activity associated with G_1 and G_2/M cell cycle arrest, while at higher doses (82.7−330.8 μM) it induced significant apoptosis. The underlying mechanism could be the xanthone-induced DNA damage, which, depending on the severity, can serve as the signal for cell-cycle arrest or apoptosis. Therefore this study suggested the potential anticancer activity of decussatin.

19.5.11 Norswertianin

The isolation of norswertianin from *S. japonica* was reported in 1969. This simple tetraoxygenated xanthone exerted significant protective action against photosensitized riboflavin-induced DNA damage in vitro (Hirakawa et al., 2005). Norswertianin and gentiacaulein potently inhibited the formation of guanosine oxidative products induced by photosensitized riboflavin, while bellidifolin and swerchirin had no substantial effects. It was speculated that the mechanism underlying their protective action was probably due to the quenching of riboflavin triplet excited state. This ability qualifies gentiacaulein and norswertianin as potential preventive agents in solar UV−induced carcinogenesis, photogenotoxicity, and phototoxicity.

In a study conducted by Uvarani et al. (Uvarani et al., 2015), norswertianin and six other xanthones isolated from *Swertia corymbosa* (Gentianaceae) were investigated for antioxidant, anti-α-glucosidase, DNA-binding, protein-binding, and cytotoxic activity. Norswertianin displayed strong antioxidant capacity due to its considerable ferric-reducing power (7206.7 ± 73.7 mmoL Fe^{2+}/g) and ability to scavenge ROS (DPPH, OH, and NO with IC_{50} values of 12.1 ± 0.8; 20.9 ± 2.9; 21.8 ± 0.7 μM, respectively). The authors presumed that high antioxidant potency of norswertianin resulted from the presence of the catechol moiety, which enhanced H-donating ability. A similar conclusion was drawn regarding its anti-α-glucosidase activity. Namely, all investigated xanthones displayed moderate anti-α-glucosidase activity, with norswertianin being the most active (IC_{50} = 23.2 ± 1.5 μM). Its activity was ascribed to the presence of free OH groups involved in H-bonding interactions with α-glucosidase. All investigated xanthones showed DNA-intercalating and bovine serum albumin-binding ability. The intercalation of xanthones into calf thymus DNA was probably determined by their planar conformation and aromatic hydrocarbon structure. Norswertianin, however, did not show any substantial cytotoxic potential against cervical, colorectal, and gastric adenocarcinoma cells.

In contrast to that, norswertianin from *Gentiana dinarica* showed significant antiglioma activity in vitro (Tovilovic-Kovacevic et al., 2018). The anti-proliferative capacity of methanol extracts from untransformed *G. dinarica* roots and roots transformed with two strains of *Agrobacterium rhizogenes* was evaluated against U251 human glioblastoma cells. The most potent activity showed extract enriched in norswertianin (17.93%) and norswertianin-1-*O*-primeveroside (14.81%). Mechanisms involved in its anticancer effect included cell cycle arrest in G_2/M phase and were accompanied by increased expression of markers for differentiated astrocytes (glial fibrillary acidic protein) and neurons (β-tubulin). Furthermore, cell differentiation was mediated through the induction of oxidative stress and Akt/mTOR-dependent autophagy. Xanthone aglycone norswertianin exerted the same effects in U251 cell culture, which strongly suggested that it was the main active principle of the investigated extract. These results proposed the xanthone-rich methanol extract of *G. dinarica* and particularly norswertianin as potential candidates for differentiation therapy of glioblastoma.

19.5.12 Gentioside

An antiparasitic property of methanolic root extract of *Gentiana kurroo* was evaluated against *Leishmania donovani* in vitro (Sidana, Kaushal, & Farooq, 2018). This protozoan parasite causes leishmaniasis, which depending on clinical form induces severe health problems. The plant extract significantly attenuated *Leishmania* promastigotes transformation (500 μg/mL; 43.1% inhibition). Searching for the active principle of *G. kurroo* roots in silico docking analysis was performed and 13 phytochemicals were docked against five drug target proteins, necessary for the *L. donovani* survival. Gentioside and norswertianolin (3,5,8-trihydroxyxanthone-1-*O*-glucoside) displayed minimum binding energies with the majority of the drug targets, which indicated the highest binding affinity to leishmanial proteins. Gentioside interacted with the active site of four protein targets, while norswertianolin was bound only to the active site of one protein. The authors have concluded that the antileishmanial activity of *G. kurroo* methanol root extract probably relies on the gentioside content.

19.5.13 Eustomin and demethyleustomin

Eustomin and demethyleustomin represent two closely structure-related polymethoxylated xanthone derivatives, eustomin is pentamethoxy-, while demethyleusomin is tetramethoxyxanthone.

Both xanthones isolated from the aerial parts of *C. erythraea*, a widely-used European medicinal herb, showed the potential to oppose the mutagenicity of different chemical agents (Schimmer and Mauthner, 1996). An ethanolic extract of *C. erythraea* significantly diminished the 2-nitrofluorene- and 2-aminoanthracene-induced mutagenicity in two strains of *Salmonella typhimurium* TA98 and T100. Eustomin significantly decreased the nalidixic acid-induced mutagenicity in strain T102, whereas it inhibited the ethyl methane sulfonate-triggered mutagenicity to a lesser degree.

Although demethyleustomin antimutagenic capacity was detected, it was less active than eustomin. Since both xanthones counteract mutagens, which act through different mechanisms, the authors assumed that investigated compounds represent antimutagens rather than desmutagens. Desmutagens directly counteract mutagen potential, while antimutagens through different DNA repair mechanisms decrease mutation frequency. In conclusion, it was speculated that eustomin acted primarily as a bioantimutagen.

Furthermore, the structures of eustomin and demethyleustomin, isolated from aerial parts of *Centaurium spicatum*, were elucidated with chemical and spectral analysis and their bioactive potential was investigated (Allam, El-Shanawany, Backheet, & Nafady, 2014). Eustomin and demethyleustomin showed strong antioxidative potential with IC_{50} values of 1.87 and 3.29 μM, respectively.

19.5.14 Corymbiferin

The bioactivities of corymbiferin, a pentaoxygenated xanthone isolated from *Gentiana corymbifera* in the 1950s (Ross, 1950), were not evaluated until the last decade. Liu et al. (Liu et al., 2013) have studied the anti-diabetic activity of corymbiferin isolated from dichloromethane extract of *Swertia bimaculata* in vitro and in vivo. The extract rich in corymbiferin increased the glucose consumption in differentiated 3T3-L1 cells by 33.16% at the dose 25 μg/mL. Furthermore, the extract (400 and 200 mg/kg, 5 weeks, i.p.) and corymbiferin (40 mg/kg, 5 weeks, i.p.) displayed significant hypoglycemic activity in STZ-treated rats fed with a diet high in fat and sucrose. Both treatments decreased blood glucose levels, lowered total serum cholesterol, low-density lipoprotein (LDL), and TG levels, and increased HDL/LDL ratio. In addition, serum insulin levels were increased, and insulin sensitivity was improved through increased expressions of IRS-2, PI3K, and Ser/Thr kinase AKT2, which are all initially downregulated in hyperglycemic rats. The extract and corymbiferin ameliorated antioxidant capacity in diabetic rats based on the recovery of SOD, catalase (CAT), glutathione peroxidase (GPx), and GSH activities in the liver of treated rats. Furthermore, histopathological examination showed a reduction in adipose tissue accumulation, as well as regeneration of damaged hepatic lobular structures and pancreatic β cells. Therefore *S. bimaculata* and its active constituent corymbiferin could be taken into account as useful unconventional agents for diabetes mellitus treatment. Moreover, corymbiferin-1-*O*-glucoside isolated from *G. amarella* has exerted an interesting activity against MAO B in vitro, inhibiting its activity by 70.5% at 10 μM concentration (Urbain et al., 2008).

19.5.15 Lanceoside

Lanceoside was first isolated from *T. lanceolatum* (Gentianaceae) in 1982 (Chun-Nan, Cheng-Hsiung, Arisawa, Shimizu, & Morita, 1982). In a study conducted by Chen et al. (Chen, Lin, Wu, & Cheng, 1993), effects of lanceoside on apomorphine- and ephedrine-induced biting behavior in rats were assessed. Previous studies have shown that xanthones with similar oxygenation patterns to lanceoside, namely lancerin (4-*C*-glucosyl-1,3,7-trihydroxyxanthone) and tripteroside (norathyriol-6-*O*-beta-D-glucoside), possessed considerable CNS modulating activity (Lin, Chiang, Arisawa, Shimizu, & Morita, 1984). Chen et al., (1993) explored the ability of xanthone-O-glycoside lanceoside to suppress stereotypic behavior induced by sympathomimetic drug ephedrine or dopaminergic agonist apomorphine. Lanceoside significantly reduced ephedrine-induced biting behavior and increased locomotor activity, with no effect on apomorphine-triggered behavioral change. These results suggested that inhibition of stereotypic behavior could be due to lanceoside potential to block central dopaminergic transmission, indicating the possible weak CNS depressant and antipsychotic activity of lanceloside.

19.6 Pharmacological activities of secoiridoids

19.6.1 Swertiamarin

One of the first studies concerning swertiamarin (SWM) bioactivity found that this active constituent of the methanol extract of *S. japonica* had anticholinergic potential. Namely, the extract (containing up to 30% of SWM) and SWM inhibited the contractile response of exposed rat colon to externally added carbachol, a potent cholinergic agent (Yamahara, Kobayashi, Matsuda, & Aoki, 1991). The authors presumed that anticholinergic action of SWM was due to the blockade of the Ach muscarinic receptors in the gut which exerted a spasmolytic effect. Overall, it is proposed that the known effectiveness of Swertia herb in treating abdominal distress is based on anticholinergic/spasmolytic properties of SWM.

19.6.1.1 Immunomodulatory, anti-inflammatory, and antioxidant activity

Saravanan et al. (2014c) investigated the immunomodulatory potential of SWM isolated from the methanol extract of *Enicostema axillare* (Gentianaceae), an Indian traditional medicine plant. Bioactivity of different doses of SWM (2, 5, and 10 mg/kg, 7 days, orally) was assessed in mice immunized with sheep red blood cells (SRBC) and changes in humoral and cellular immunity were tracked. SWM induced a dose- and time-dependent rise in the hemagglutinating antibody titer and a dose-dependent rise in the number of antibody-secreting cells in the spleens of immunized animals. The previous results indicated the enhancement of the humoral immune response in immunized mice treated with SWM. Further, SWM diminished the response to a delayed-type hypersensitivity reaction, with simultaneous attenuation of elevated proinflammatory cytokines serum levels (IL-1β and TNF-α). Moreover, in vitro studies showed that SWM decreased mRNA and protein expression of IFN-γ, and increased mRNA and protein expression of IL-10 and IL-4 in concanavalin A-treated splenocytes. Thus SWM reduced the Th1-mediated proinflammatory (IFN-γ) and increased Th2-mediated anti-inflammatory (IL-10, IL-4) response. In addition, SWM attenuated mRNA and protein expression of proinflammatory molecules (IL-1β, TNF-α, and IL-6) in LPS-activated macrophages, as well as the parameters of phytohemagglutinin-induced neutrophils activation (adhesion ability, superoxide formation, myeloperoxidase release). SWM anti-inflammatory potential was confirmed in silico, too. Overall, this study suggested that SWM possessed the potential to modulate humoral and cell-mediated immunity.

Moreover, Saravanan et al. (2014b) pursued the anti-inflammatory potential of SWM, isolated from *E. axillare*, in a model of chronic arthritis. Rheumatoid arthritis (RA) is a chronic inflammatory disease, featuring ongoing progressive cartilage and bone devastation. The primary effectors of cartilage destruction are invasive fibroblast-like synoviocytes (FLS), characterized by extensive production of proteases (Firestein, 2009). The anti-inflammatory potential of SWM was evaluated in the culture of FLS isolated from Freund's complete adjuvant (FCA)-induced arthritic rats treated with IL-1β. The FLS treatment with different doses of SWM (50−1000 μg/mL) did not affect cellular viability. In contrast, IL-1β-induced FLS proliferation and NO production were in a dose-dependent manner blocked with SWM treatment (50 μg/mL). Further, increased expression of caspase-3, the set of proinflammatory molecules (TNF-α, IL-6, PGE2, COX-2, iNOS, matrix metalloproteinase (MMP)), and receptor activator of nuclear factor κB ligand (RANKL) was reduced both on the mRNA and protein levels with SWM treatment. In addition, SWM in a dose-dependent manner downregulated IL-1β-induced increased expression of p38 MAPK, a molecule positively correlated with bone destruction in RA. From previous findings, the authors concluded that the anti-arthritic capacity of SWM is due to the attenuation of inflammatory mediators and bone destructing markers in IL-1β-triggered FLS.

Further, the anti-inflammatory potential of SWM was evaluated in rats treated with FCA to induce chronic arthritis (Saravanan et al., 2014a). The SWM (2, 5, 10 mg/kg b.w.) notably attenuated arthritis-induced paw thickness, the marker of arthritis severity. Concordantly, the body weight of STM-treated arthritic animals was preserved. The levels of plasma and liver lysosomal enzymes which degrade proteoglycan in the cartilage (acid phosphatase, β-D-glucuronidase, *N*-acetyl-β-D-glucosaminidase, β-D-galactosidase, and cathepsin D) were restored to the levels measured in the control group of animals, after applying the highest dose of SWM. These results indicated that SWM possessed potential to abolish the effect of toxic molecules that are the hallmark of arthritis. The more detailed examination of molecular mechanisms underlying SWM protective effect against the adjuvant-induced arthritis showed SWM's capacity to modulate the inflammatory response. Namely, SWM affected adjuvant-induced release of proinflammatory cytokines (IL-1, TNF-α, IL-6) and proangiogenic enzymes (MMPs, iNOS, PGE2, PPAR-γ, and COX-2), which synergistically promote synovial inflammation. In concordance with the latter, SWM augmented the levels of anti-inflammatory proteins (IL-10, IL-4) compared to the adjuvant-induced animals. SWM opposed to the release of NF-κB/IκB and JAK2/STAT3 activated by adjuvant, both in animals and in cultured LPS-induced RAW 264.7 macrophage cells. In silico analysis showed SWM's potential to dock with proinflammatory enzymes. The results propose that SWM could be an anti-inflammatory and antirheumatoid agent.

Another study showed both the anti-inflammatory and antioxidative potential of hydroethanolic extract from *S. chirayita* leaves against FCA-induced arthritic rats (Lad and Bhatnagar, 2016). The extract orally administered (200 mg/kg b.w., per day) significantly reversed elevated lipid peroxidation and protein oxidation in arthritic animals. Similarly, the compromised intracellular antioxidant defense system (SOD, CAT, glutathione-S-transferase (GST), and GSH) in arthritic rats, were restored after treatment with the extract. The extract exerted anti-inflammatory potential since it attenuated raised serum proinflammatory cytokines (TNF-α and IL-1a) and paw edema of the arthritic rat. Marked reduction of changes in arthritic ankle joints was detected using histological and radiographic analysis. Finally, HPLC analysis revealed SWM and amarogentin as extract key phytoconstituents. Interestingly, the extract antioxidant potential was confirmed in vitro, and it was in a positive correlation with the phytoconstituents.

SWM from the ethyl acetate extract of *E. axillare*, showed a similar anti-edematogenic effect on paw edema caused by inflammatory agents—carrageenan, formalin, and histamine in rats (Vaijanathappa and Badami, 2009). Oral administration of SWM (100 and 200 mg/kg b.w.) generated a powerful dose-dependent attenuation of carrageenan-induced paw edema. Significantly, no dose-dependent activity of SWM was observed in the formalin-induced edema. SWM exerted significant inhibition in the histamine-triggered paw edema, after 6 and 24 h. The authors noted that the activity of SWM was superior in comparison to standard diclofenac sodium at all times. In addition, considering the obtained results, SWM possesses anti-edematogenic potential, and the anti-inflammatory activity of *E. axillare* could be attributed to SWM.

The remarkable antioxidant potential of SWM from *E. axillare* was detected in vitro (Vaijanathappa and Badami, 2009). The best activity was determined using ABTS and hydrogen peroxide methods, with IC_{50} values of 2.83 and 5.70 μg/mL, respectively. Only modest capacity, with IC_{50} values of 52.56 and 78.33 μg/mL, was observed in hydroxyl radical scavenging by deoxyribose and lipid peroxidation methods, respectively. Additionally, the total antioxidant capacity was found to be 4.51 mM of ascorbic acid per gram of SWM. The results clearly point out the antioxidative potential of SWM.

19.6.1.2 Hepatoprotective activity

The protective potential of SWM, a secoiridoid natural compound, against CCl_4-induced hepatotoxicity in rats, was investigated by Wu et al. (Wu, Li, Li, & Song, 2017). The treatment of animals with SWM alone had no effect on hepatic cellular architecture, while CCl_4 induced extensive histopathological changes, as well as hepatic fibrosis. SWM significantly reduced both histopathological lesions and hepatic fibrosis induced by CCl_4 indicating SWM potential to attenuate the intensity of liver damage. Further, SWM reduced the degree of ALT, AST, and ALP upregulated in animals with hepatic injury. The oxidative stress in liver induced with CCl_4 was suppressed by SWM since it attenuated the levels of MDA, the marker of lipid peroxidation. In accordance, SWM increased liver antioxidative capacity through elevation of SOD, GPx, and GSH levels. Furthermore, SWM decreased neutrophil infiltration in hepatic lesions, iNOS, and NO levels, and the level of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in CCl_4-treated rats, implying SWM anti-inflammatory potential. In this study, the effect of SWM on detoxification enzymes and signaling molecules involved in maintaining antioxidant homeostasis, Nrf2/heme oxygenase-1 (HO-1) pathway in the liver, was followed. Western blot analysis indicated that SWM restored CCl_4-impaired expression of cytochrome P450 (CYP) 2E1 and 3A, the well-known liver detoxification enzymes and significantly upregulated the expression of Nrf2/HO-1. In conclusion, the authors suggested that SWM counteracted CCl_4-induced liver injury based on its anti-inflammatory and antioxidant properties, and the potential to strengthen the detoxifying power of liver cells. In agreement with the latter study, Zhang et al. (Zhang, Chen, Wu, & Song, 2019) observed SWM's potential to oppose CCl_4-induced liver injury, too.

In addition, the hepatoprotective potential of methanol extracts from *Gentiana asclepiadea* aerial parts and roots against CCl_4-induced liver damage in animals was also detected (Mihailović et al., 2013). Phytochemical investigation of *G. asclepiadea* methanol extracts determined a high concentration of swertiamarin in doses of 24.805 ± 4.039 and 5.739 ± 0.614 mg/g of extracts, respectively (Mihailović et al., 2013). In the extracts, the presence of sweroside and gentiopicrin was observed, too. The iridoid-rich extracts exerted the decrease in the level of liver injury markers (e.g., serum transaminases, alkaline phosphatase, and total bilirubin) and augmented the degree of total protein. Concurrently, the extracts induced a remarkable increase in the levels of CAT, SOD, and GSH, followed by an obvious decrease in the levels of MDA, suggesting an antioxidative potential of iridoid−rich extracts. The histopathology of rat liver confirmed that extracts counteracted liver injuries, too. The gained results advocate an antioxidative and hepatoprotective effect of SWM-rich extracts against liver damage induced by CCl_4.

Furthermore, SWM exerted the protective effect against liver injury induced by intraperitoneal administration of D-GalN, mimicking viral hepatic damage, in rats (Jaishree and Badami, 2010). For this study, SWM from ethyl acetate extract of *E. axillare* (Gentianaceae) whole plant was evaluated for antioxidant and hepatoprotective capacity. Before the induction of liver injury, SWM was orally administrated to animals for 8 days. SWM restored all D-GalN-induced disturbances in biochemical liver injury parameters (AST, ALT, ALP, TG, total cholesterol, total bilirubin, creatinine, total protein, and albumin). In addition, SWM showed potent antioxidant property, it triggered an increase in CAT, SOD, and GSH simultaneously with reduced levels of lipid peroxidation in serum, liver, and kidney. The histopathological analysis showed reduced hepatic damage in D-GalN + SWM-treated animals compared to the D-GalN group. Similarly, SWM restored a normal histological appearance of kidney in animals with hepatic injury. A possible mechanism of the hepatoprotection afforded by SWM thus might be due to its antioxidant action.

In accordance with previous studies, the crude ethanol extract of total iridoids and xanthones (TIXS), SWM, and swertianolin exhibited the hepatoprotective potential against liver damage triggered by α-naphthylisothiocyanate. The fingerprint chromatography of TIXS, extracted from *S. mussotii* showed that among 12 identified compounds SWM and swertianolin were the most abundant (Tian, Zhang, Wang, Shan, & Jiang, 2014). In concordance with previous studies, elevated biochemical markers of liver injury in sera of animals were significantly lower in rats treated with TIXS, SWM, and swertianolin. All tested compounds increased the bile flow in animals with hepatic injury, too.

Dai et al. (2018) assumed that activation of human cytochrome P450 isoform 3A4 can be the main mechanism for reducing liver toxicity induced by aconitum (Negoro et al., 2016; Yu et al., 2016), a plant used for antipyretic, analgesic, antirheumatic treatments mainly in traditional medicine (Wang et al., 2006). To gain the binding efficiencies of vast natural compounds to the active sites of human cytochrome P450 isoforms the molecular docking analysis was performed, and after comprehensive evaluation SWM and six other compounds were selected for further analysis (Dai et al., 2018). SWM possessed the least potent binding affinity among all six examined compounds, and in accordance, it was the least efficient to upregulate CYP3A4 mRNA levels. Further analysis of hepatoprotective potential was done with amarogentin, which exhibited the highest docking score, and the most potently enhanced expression levels of CYP3A4 mRNA in HepG2 cells.

19.6.1.3 Hypoglycemic and anti-diabetic activity

Several studies describe the beneficial effects of SWM on diabetes mellitus, the chronic metabolic condition characterized by the presence of chronic hyperglycemia. The effects of aqueous extract of *Enicostemma littorale* (1 g/kg) and SWM (50 mg/kg) on diabetic nephropathy, one of the common complications of diabetes, were investigated (Sonawane et al., 2010). The extract and swertiamarin had no effect on the loss of body weight and food intake in STZ-treated diabetic rats. However, applied treatment notably decreased the elevated water intake. Increase in serum glucose, cholesterol, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase, as well as creatinine and urea levels, biochemical markers of the nephropathy in rats, detected in diabetic animals were significantly reduced in extract- and SWM-treated animals. Since glomerular hypertrophy and glomerular injury are diabetic nephropathy hallmarks, the effect of SWM and extract-treated animals was examined on kidney histopathological sections. Overall results showed the potential of both extract and SWM to repair nephrotoxic-induced damages in diabetic animals.

Swertiamarin (75 mg/kg/day, i.p.) remarkably decreased serum glucose, TG, nonesterified free-fatty acid, and cholesterol levels in Zucker fa/fa rats, an animal model of genetic obesity (Vaidya, Giri, Jain, & Goyal, 2012a). In addition, SWM restrained the rise of urea levels in Zucker fa/fa rats. The SWM reduced the serum levels of matrix metalloproteinases 3 and 9 (MMP), proteolytic enzymes with clinical significance in diabetes and diabetic complications (Derosa et al., 2007). The reported data suggested that SWM-induced downregulation of serum MMP-9 and MMP-3 could be one of the presumed mechanisms for the beneficial effects of SWM on cardiovascular complications in diabetes. Additional research revealed gentianine, an active metabolite of swertianin, as a molecule responsible for the antidiabetic effect of SWM since it significantly increased adipogenesis, which is associated with the upregulation of PPAR-γ, GLUT-4, and adiponectin mRNA expression (Vaidya, Goyal, & Cheema, 2013).

It was found that the fraction from the methanol extract of *Enicostemma hyssopifolium* (Gentianaceae) aerial parts contains SWM and swertisin, and its influence on cells and systemic glucose homeostasis was further examined (Patel and Mishra, 2011). The authors detected that the fraction and both isolated compounds inhibit aldose reductase; SWM exhibited the highest IC_{50} value of 7.59 μg/mL. The fraction and swertisin inhibited α-glucosidase and displayed strong cytoprotective capacity against STZ-induced toxicity in rat insulinoma RINm5F cell line. On the other hand, SWM neither affected the α-glucosidase activity nor STZ-induced RINm5F cell death. In the insulin release study, SWM showed strong insulin-secreting activity, both in the glycemic and hyperglycemic condition in RINm5F cell culture, whereas swertisin and fraction significantly increased insulin levels only in the hyperglycemic milieu. Contrary to in vitro studies, SWM had no influence on insulin secretion in vivo. The authors speculated that swertisin is most probably responsible for detected hypoglycemic bioactivity of *E. hyssopifolium* methanol extract.

Dhanavathy (Dhanavathy, 2015) did one of the most comprehensive studies of SWM anti-hyperglycemic activity. The focus of this investigation was assessing the hypoglycemic effect of SWM, isolated from leaves of *E. littorale*, as well as to estimate histopathological alterations in the pancreas, liver, kidney, and heart of SWM-treated diabetic rats. Firstly, the potential toxicity effect of SWM was investigated. The animals were treated orally with different doses ranging from 100 to 1000 mg/kg body weight, and neither lethality nor physiological, behavioral, and neurological shifts were detected. Treatment of STZ-induced hyperglycemic rats with different doses of SWM (15, 25, 50 mg/kg b. w., p.o.) for 28 days resulted in a potent reduction in fasting blood glucose, HbA1c, total cholesterol, TG, LDL, and

upregulation of the levels of hemoglobin, plasma insulin, total protein, body weight, and HDL levels compared to STZ-treated animals. In addition, histopathological analysis of SWM-treated diabetic pancreas demonstrated regeneration of Langerhans islets, as well as reduction of degenerative changes in the hepatocytes, glomeruli, and cardiac myocytes compared to the diabetic rats. The authors concluded that SWM possessed hypoglycemic, hypolipidemic, and cytoprotective activity. A broad spectrum of anti-diabetic mechanisms indicates SWM to be a compound with strong potential for treating diabetes and related complications. Hence, it can be established as a powerful oral anti-diabetic drug.

Also, swertiamarin showed a beneficial effect on dyslipidemia in STZ-induced type 2 diabetic rats (Vaidya et al., 2012b). Dyslipidemia represents one of the major risk factors for the development of cardiovascular complications in diabetes mellitus and it is attributed to an increase in free fatty acid flux, secondary to insulin resistance. Similar to previous studies, SWM (50 mg/kg, once a day, 6 weeks) remarkably decreased serum TG, cholesterol, and LDL levels in diabetic animals. Concurrently with a decrease in serum fasting glucose, the rise in insulin sensitivity index in SWM treated animals was detected. The results indicated a beneficial effect of SWM on dyslipidemia, a diabetic complication.

E. littorale is widely used for treating the symptoms of both types 1 and 2 diabetes (Patel et al., 2013). Swertiamarin from *E. littorale* showed the potential to regulate the level of serum glucose, insulin, and lipid profile in experimentally triggered diabetes mellitus type 2 in rats (Patel et al., 2013). Since SWM reversed activity of G6Pase and HMG-CoA reductase to homeostatic values and modulated transcription of numerous genes involved in the maintenance of glucose and lipid levels (PEPCK, GK, Glut 2 transporter, PPAR-γ, leptin, adiponectin, LPL, SREBP-1c, and Glut 4), the authors concluded that SWM increased insulin sensitivity and regulated carbohydrate and fat metabolism.

19.6.1.4 CNS modulating activity

Iridoids and iridoid-containing plants have been reported to possess certain effects on the central nervous system (CNS). A methanol extract of *G. lutea* roots remarkably prolonged mobility time in the swimming endurance test and showed minor analgesic capacity (Oztürk, Başer, Aydin, Oztürk, & Caliş, 2002). Subsequently, HPLC analysis of methanol extract showed that it contained gentiopicroside, SWM, and sweroside (10.5%, 1.8%, and 0.9%, respectively). It could be concluded that the effects of the methanol extract could be ascribed to these three secoiridoid compounds.

Moreover, it is described that SWM from an extract of *Gardenia fructus* had neuritogenic capacity in rat pheochromocytoma cells, subclone PC12h cells (Chiba, Yamazaki, Kikuchi, Kakuda, & Kikuchi, 2011). Namely, treatment with SWM (100 μM, 2 days) induced significant neurite outgrowth in PC12h cells, while no toxicity was detected. The results indicate that SWM could be a promising molecule for the development of neurotrophic factor-like compounds.

Further, Deng et al. (2017) investigated a potential anticonvulsant activity of SWM in pilocarpine-treated mice. Pretreatment of mice with valproate sodium and different doses of SWM (50, 150, and 450 mg/kg) drastically delayed the onset of the first convulsion, and the incidence of status epilepticus and mortality were reduced. The presence of two higher doses of SWM attenuated the neuronal damage, and significantly inhibited astrocytic activation. The highest concentration of SWM downregulated the expressions of proinflammatory cytokines (IL-1β, IL-6, TNF-α), while the expression of anti-inflammatory IL-10 was upregulated. The results indicate SWM as a potentially important adjuvant in anticonvulsant therapy.

SWM, isolated from *G. macrophylla*, showed a neuroprotective effect on cerebral ischemia/reperfusion injury in vivo and in vitro (Wang et al., 2019). Different doses of SWM were applied to the animals before middle cerebral artery occlusion, and attenuation in infarct volume, the number of apoptotic neurons, oxidative damage, together with neurologic recovery was observed. The mechanism underlying the protective effect of SWM (0.1, 1 or 10 μM) was investigated in the oxygen−glucose deprivation/reperfusion model in primary hippocampal neurons. SWM diminished the level of ROS and increased cell viability in vitro. It is demonstrated that the pretreatment with SWM induced nuclear translocation of Nrf2 and enhanced the expressions of NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1 both in vivo and in vitro. The results shown above indicated that SWM could be considered as a promising compound with a protective effect against cerebral ischemia/reperfusion injury.

19.6.2 Gentiopicroside (gentiopicrin, GP)

Due to space limitations, only a few bioactivities of gentiopicroside (GP) will be discussed in this chapter. Gentiopicroside is a bitter secoiridoid glycoside abundantly present in the Gentianaceae.

19.6.2.1 Hypoglycemic and anti-diabetic activity

Hypoglycemic and anti-diabetic activity of GP was investigated in vitro (Huang, Li, Mei, & Chen, 2016) and in vivo (Yang, Chen, Li, Hui, & Gao, 2018b). As mentioned earlier, GP and sweroside isolated from *Veratrilla baillonii* (Gentianaceae) ethanol extract showed a strong insulin-like effect on Pck1 gene expression and activated insulin transduction pathway components—Akt S473 and ERK in HL1C hepatoma cells (Huang et al., 2016). Gentiopicroside and geniposide, another secoiridoid glycoside, displayed similar effects on L02 human liver cell line (Yang et al., 2018a, 2018b). These two glycosides, at a concentration of 100 μM, significantly promoted glucose consumption in L02 liver cells. Importantly, the effect was more pronounced in insulin resistant (IR) than in normal L02 cells. Similarly to the anti-diabetic drug metformin, compounds (100 μM) decreased glucose production in IR cells. Moreover, secoiridoids significantly downregulated transcription and expression levels of glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PEPCK), key cell positive regulators of glucose production. These effects were most probably mediated through PI3K-Akt-dependent phosphorylation of FOXO1 and subsequent reduction of its transcriptional activity. Nuclear translocation of FOXO1, which is a step necessary in FOXO1 activation, was also suppressed by secoiridoids. More importantly, the hypoglycemic action of GP and geniposide was demonstrated in vivo. Both investigated compounds after 2 weeks of administration (100 mg/kg, i.p.) to the high-fat diet-fed mice strongly reduced fasting blood glucose, improved glucose tolerance, and suppressed transcription of G6PC and PEPCK genes in mice liver. Overall, this study indicates a potential application of iridoid glycosides in diabetes treatment.

19.6.2.2 CNS modulating activity

The first study that implied CNS's modulating potential of secoiridoids showed considerable antidepressant and slight analgesic activity of methanolic extract of *G. lutea* roots containing GP, SWM, and SWS in mice (Oztürk et al., 2002). A single application of extract (250 and 500 mg/kg, i.p.) remarkably increased endurance in mice swimming capacity. Moreover, no obvious influence on general behavior or lethality was detected. The observed effects were not dose-dependent, which led authors to conclude that different components, even presented at trace concentrations in the extract, may have antagonistic actions. Additionally, it was concluded that CNS stimulant and/or adaptogenic (anti-stress) capacity of the extract could be ascribed to the metabolism activation by extract. The latter presumption was based on a long history of traditional use of gentian root as a tonic for appetite and digestion improving. Altogether, the authors suggested that CNS modulating activity could be attributed to secoiridoid components present in the methanol extract.

Following this, several studies have confirmed antinociceptive (Chen et al., 2008; Liu et al., 2016; Liu et al., 2014) and antidepressant activity (Deng, Zhao, Xu, Jin-Hou, & Li, 2018; Liu et al., 2014) of GP in various model systems. Thus GP exerted analgesic effects on inflammation-related mechanical allodynia (central pain sensitization following normally nonpainful repetitive stimulation) (Chen et al., 2008). Inflammation was provoked by s.c. injection of Freunds complete adjuvant (FCA) in hind-paw of mice (Chen et al., 2008) and GP was administrated (50, 100, and 200 mg/kg, twice a day, 3 days i.g.). Peripheral inflammation increased the expression of NR2B subunit of glutamate *N*-methyl-D-aspartate (NMDA) receptor in the anterior cingulate cortex (ACC), the forebrain structure known for pain-related information processing. Gentiopicroside downregulated FCA-increased expression of the NR2B receptor subunit and reversed NR2B receptor-related postsynaptic currents in the ACC. These results suggested that GP analgesic capacity was partially due to inhibition of NR2B receptors function in the ACC area. Further, to explore the possible mechanism of NR2B-inhibition, GP's potential to modulate cAMP levels was assessed. Adenylate cyclases, the enzymes catalyzing the conversion of ATP into cAMP, are important mediators of synaptic transmission at ACC synapses in mice with chronic pain. Expectedly, GP in a dose-dependent manner reversed FCA-elevated cAMP levels in the ACC area of treated mice and on brain slices. Concurrently, no significant effect on the basal excitatory synaptic transmission or GABAergic (γ-aminobutyric acid) transmission in the ACC was detected. The obtained results clearly demonstrate the analgesic effects of GP in persistent inflammatory pain and offer a possible explanation for traditional herbal use in pain treatment.

Gentiopicroside, due to the ability to modulate expression and activity of NR2B subunit of glutamate NMDA receptor in different parts of the brain, and to concomitantly affect other neurotransmitter systems in CNS, exerted additional CNS-related pharmacological activities in vivo. Namely, it showed the ability to reverse morphine-induced rewarding activity (morphine dependence model) (Liu et al., 2012) and to attenuate reserpine-induced pain and depression in mice (Liu et al., 2014). In the former study (Liu et al., 2012), morphine dependence was induced by chronic morphine administration (10 mg/kg, 7 days, s.c.) and effects of GP (100 mg/kg, twice a day, 8 days, i.p.) on rewarding behavior and receptors levels of NMDA and dopamine, well-known mediators of morphine dependence, were followed. The changes

in receptor levels were tracked in nucleus accumbens (NAc), a forebrain structure with a role in drug addiction. Gentiopicroside significantly reduced GluN2B-NMDA subunit and D2 receptor expression in the NAc of morphine-dependent mice (Liu et al., 2012). Likewise, GP partially downregulated excitatory synaptic AMPA and NMDA transmission enhancement in the NAc caused by chronic morphine administration. Gentiopicroside, however, did not inhibit morphine-induced hyperlocomotion. The ability to affect the morphine-induced neurotransmitter changes in the brain indicates GP to be a potentially useful compound in attenuation of morphine-dependence. In the latter study (Liu et al., 2014), reserpine (1 mg/kg s.c. daily for 3 days) was used to cause depressive-like behavior and decrease in the nociceptive threshold in mice due to its ability to deplete endogenous catecholamine (norepinephrine (NE), dopamine (DA), and serotonin (5-HT)) stores from nerve endings. Gentiopicroside (50, 100, and 200 mg/kg, twice a day, 3 days, i.g.) in a dose-dependent manner increased paw withdrawal latency (PWL) to heat stimuli in reserpinized mice, indicating an increased pain threshold in GP-treated animals. The investigated secoiridoid also dose-dependently decreased immobility time in the forced swimming and tail suspension tests in reserpinized mice, suggesting antidepressant-like effects of GP. These behavioral parameters were accompanied by attenuation of oxidative stress parameters (MDA, CAT) and downregulation of GluN2B-NMDA subunit expression in basolateral amygdala of reserpinized animals by GP. Considering that amygdala is a brain region responsible for the coordination of negative emotional responses to threatening stimuli, concentrations of the monoamine neurotransmitter in the basolateral amygdala (BLA) were assessed. Gentiopicroside restored a reserpine-induced decrease in 5-HT, NE, and DA levels, indicating that antidepressant and analgesic effect of gentiopicroside involved the modulation of BLA monoaminergic neurotransmitter systems. Additionally, GP reversed altered levels of apoptosis-related proteins (caspase 3, Bcl2) in the BLA of reserpinized mice, an action that was probably mediated through attenuation of NMDA glutamate receptor-triggered excitotoxicity. The antidepressant ability of GP was proved in LPS-induced depressive-like behavior in mice as well (Deng et al., 2018). Namely, in addition to altered neurotransmitter levels, neuroinflammatory disturbances are also connected with depressive disorder in humans. Gentiopicroside (50 mg/kg, once a day, 3 days, i.p.) prevented the development of depressive-like behavior induced by LPS (0.5 mg/kg, acute administration, i.p.) through the restoration of LPS-induced alterations of inflammatory mediators (IL-1β and TNF-α) in plasma and brain. It also prevented overactivation of indoleamine 2,3-double oxygen enzyme (IDO), which catalyzes tryptophan degradation in the brain causing the formation of NMDA agonist quinolinic acid and subsequent NMDA receptor activation. The elevated NMDA transmission in the prefrontal cortex of mice challenged by LPS was recovered by GP through the reduction of increased expression levels of GluN2B-containing NMDA receptors. These studies highlighted the multifaceted antidepressant potential of GP and offered a new therapeutic approach for the treatment of depression.

19.6.2.3 Hepatoprotective activity

For the treatment of hepatic and biliary diseases in Chinese traditional medicine two plants are used: *Gentiana rigescens* and *G. macrophylla*. Several studies suggested GP to be one of the potentially most active components in both plants. To address GP capacity to oppose cholestasis, mice were pretreated for 5 days with iridoid (130 mg/kg, i.g.), and cholestatic liver injury was induced with a single dose of α-naphthylisothiocyanate (ANIT, 75 mg/kg, i.g.) (Tang et al., 2016). In cholestatic animals, a dramatic increase of ALT, ALP, TBA, and TBIL in serum, and accumulation of numerous biochemical markers of cholestasis in serum and the liver, were observed. In addition, in animals with cholestatic liver injury, gene expression of bile acid synthesis related genes was attenuated, while the expression of bile acid transporters in liver was increased. The treatment with GP blocked ANIT-triggered acute cholestasis and liver injury and regulated disturbed homeostasis of bile acids. The more detailed analysis of mechanism on the basis of ANIT-induced cholestasis revealed 73 metabolites and 84 proteins, regulating the metabolism of bile acids, fatty acids, and glycerophospholipids, involved in cholestasis dysfunction (Han, Xu, Xiong, Zhang, & Wang, 2018). Gentiopicroside exhibited an ability to recover shifts in protein expression and mRNA level toward homeostasis. Overall results indicate GP as one of the potential powerful drugs for the treatment of cholestasis.

Furthermore, the extracts from *Gentianella turkestanerum* showed hepatoprotective potential against CCl_4-triggered liver injury in mice (Yang, Zhu, Ju, Jiang, & Hu, 2017). In agreement with previous studies, CCl_4 induced the upregulation of hepatic injury biomarkers (AST, ALT, ALP, TP, TB, SOD, MDA, GSH, and CAT). The treatment with *G. turkestanerum* extracts (butanol, water, ethyl acetate, 100−400 mg/kg, via gavage) reversed observed changes. Although all extracts showed hepatoprotective potential, the most potent against liver injury was butanol extract. The high content of SWM, GP, and SWS was determined in butanol extract by HPLC. In addition, the methanol secoiridoid-rich extracts (SWS, SWM, and GP) of *Gentiana cruciata* (aerial parts and roots) showed similar hepatoprotective potential against CCl_4-induced liver injury in rats (Mihailović et al., 2014).

Gentiopicroside from the butanol extract of *S. japonica* showed mild protective capacity (25−50 mg/kg) against liver injury induced with D-GalN/LPS, while the extract itself exhibited potent activity (Hase et al., 1997). The more recent study showed D-GalN/LPS-triggered increase in aminotransferase, lipid peroxidation, and TNF-α were abolished with GP pretreatment (Lian et al., 2010). In agreement, GP recovered decline in a GSH content in mice with hepatic injury. The antiapoptotic potential of GP was revealed since secoiridoid remarkably suppressed the markers of apoptosis (caspase-3 cleavage, PARP cleavage, cytosolic cytochrome c release, and DNA fragmentation) triggered with D-GalN/LPS. Accordingly, GP opposed the disruption of Bax/Bcl-2 protein ratio in mice with liver injury. The mechanism of GP hepatoprotective effect involved, at least partly, modulation of JNK and ERK pathways.

Furthermore, it was shown that GP, approved for the treatment of acute jaundice and chronic active hepatitis in China, modulated the activity of cytochrome P450 (CYP450). In the study of Deng et al. (2013), the potential of GP to interact with different isoforms of CYP450 in vitro was assessed. Gentiopicroside inhibited in a dose-dependent manner CYP2A6 and CYP2E1 in human liver microsomes, with IC_{50} values of 21.8 and 594 μg/mL, respectively (Deng et al., 2013). An IC_{50} value for CYP2A6 was similar to the peak serum concentration that GP can achieve in the body, indicating a possibly relevant clinical interaction. The high concentration of GP induced a remarkable rise of CYP1A2 and CYP3A4 activity in vitro. In contrast, no significant induction of CYP1A2, CYP3A4, or CYP2B6 isoforms in cultured human hepatocytes was observed. The authors emphasized that CYP2A6 inhibition induced by GP should be additionally examined in vivo.

19.6.2.4 Anti-inflammatory activity

Among a variety of pharmacological activities, GP exhibited a protective effect toward pulmonary fibrosis, chronic and ultimately fatal interstitial lung disease (Chen et al., 2018). Severe pulmonary fibrosis and inflammation induced by bleomycin in mice were attenuated with GP treatment (2.5 and 10 mg/kg, i.p., once a day, 28 days). Gentiopicroside reversed histopathological changes in mice lung tissue triggered by bleomycin. Furthermore, GP displayed anti-inflammatory potential, since it significantly depressed the degree of proinflammatory molecules, TNF-α and IL-1β, in bronchoalveolar lavage fluid. Accordingly, the expression of profibrotic cytokines TGF-β1 and CTGF (connective tissue growth factor) in the lungs of pulmonary fibrosis mice was downregulated. The content of hydroxyproline in the lungs of bleomycin-treated mice was reduced when GP was administrated. Moreover, in vitro studies showed GP potential to block the epithelial-mesenchymal transition of A549 cells stimulated by TGF-β1, in a dose-dependent manner. Overall, the results advocate GP as a potential drug candidate for pulmonary fibrosis.

The pharmacological activity of GP involves anti-inflammatory potential in various conditions. The ability of GP to suppress inflammation in dextran sulfate sodium (DSS)-induced colitis in a mouse model was investigated by Niu et al. (Niu et al., 2016). The oral administration of GP (200, 100, and 50 mg/kg) attenuated DSS-triggered loss of body weight, diarrhea, the extent of a colonic mucosal injury, histological changes, and modulated the activity of myeloperoxidase (MPO) in the colon. The possible mechanism underlying GP protective capacity involves, at least partly, its suppressive potential on the levels of TNF-α, IL-1β, IL-6, COX-2, and iNOS. The obtained results indicate a good capacity of GP for colitis treatment.

Furthermore, GP exhibited anti-inflammatory effect on acute pancreatitis in rat induced by sodium taurocholat, and it is probably due to its potential to inhibit the release of inflammatory mediators TNF-α and IL-1β, and NF-κB p65 protein expression (Lv, Gu, & Chen, 2015). The orally administrated GP opposed the sodium taurocholat-evoked increase of serum amylase and lipase activity, as well as pancreatitis-induced decrease in the pancreas mass/body mass index and tissue water content.

In contrast to previous studies, GP, isolated from the roots of *Gentiana triflora*, had no effect on LPS-triggered expression of TNF-α in RAW264.7 cells (Yamada, Kikuchi, Inui, Takahashi, & Kimura, 2014). The authors indicated gentiolactone to be an active principle of the extract. Similarly, GP showed only a moderate inhibitory effect in LPS-induced production of NO in RAW 264.7 (Wang, Xu, Jiang, & Zhang, 2013).

19.6.3 Sweroside

The bioactivities of sweroside (SWS) are extensively studied since it is one of the three most characteristic secoiridoid glycosides occurring in Gentianaceae species (Jensen & Schripsema, 2002).

19.6.3.1 Hepatoprotective and anti-inflammatory activity

As previously mentioned, the traditional use of Gentianaceae plants is based on their characteristic bitter taste, which originates from secoiridoids. Owing to that, gentian root is used in many countries for stomachache and for treatment of gallbladder and liver diseases (Šavikin et al., 2015). Thus it is not surprising that one of the first evaluated and reported activities of SWS was hepatoprotective (Jie, Yaping, & Klaassen, 1994). Sweroside (100 mg/kg) and six other compounds/mixtures, all used in traditional Chinese medicine, were administered s.c. for 3 days to mice and liver injury was triggered using CCl_4, acetaminophen (AA), cadmium (Cd), and allyl alcohol (ALOH) (Jie et al., 1994). Sweroside decreased CCl_4- and Cd-induced elevation in serum activities of ALT and sorbitol dehydrogenase (SDH) and reduced histopathological features of hepatic necrosis. However, no alleviation in hepatotoxicity induced by AA and ALOH was detected, and it was concluded that the hepatoprotective effect of SWZ is only mild. A similar finding was obtained in a study of the hepatoprotective ability of *S. japonica* butanol extract against inflammatory liver injury triggered by coadministration of D-galactosamine (D-GalN) and LPS in vivo (Hase et al., 1997). The activity-guided fractionation of butanol extract led to the isolation of tetrahydroswertianolin, xanthone derivative, and two iridoids—GP and SWS. The isolated compounds were applied s.c. (25 and 50 mg/kg, twice a day before injury onset) and the most potent compound in decreasing elevated ALT levels was tetrahydroswertianolin, while SWS and GP showed only moderate activity. In contrast, a significant number of studies since then have shown that secoiridoid-rich extracts derived from different plants (*G. asclepiadea*, *G. cruciata*, *G. turkestanerum*) afforded protection against CCl_4-induced hepatotoxicity (Mihailović et al., 2013, 2014; Yang et al., 2017), implying the important role of these bitter secondary metabolites in hepatoprotection. Although the exact mechanism of action has not been identified yet, some studies suggest that secoiridoids could enhance antioxidant defense through increased activities of antioxidant enzymes SOD and CAT in the liver, or by increasing hepatic GSH levels (Mihailović et al., 2014). On the other hand, the ability of secoiridoids to, at least partly, relieve inflammatory liver injury could be a consequence of their anti-inflammatory effect. Indeed, in vitro and in vivo studies have shown a significant anti-inflammatory potential of SWS and its derivatives (He et al., 2015; Yang et al., 2016). Sweroside derivatives inhibited NO and IL-6 production in LPS-treated RAW264 macrophages (He et al., 2015). Accordingly, a 5-day administration of SWS (120 mg/kg, intragastric) decreased hepatic mRNA and protein expression of proinflammatory cytokines, including TNF-α, IL-6, mKC (mouse keratinocyte derived factor), MIP-2 (macrophage inflammatory protein 2), and ICAM-1 (intercellular adhesion molecule-1) triggered by ANIT (Yang et al., 2016).

The anti-inflammatory potential of SWS raises the possibility of its application in several inflammation-based diseases. For example, SWS has shown protective action in IL-1β-stimulated rat articular chondrocytes, implying a possible ability to suppress osteoarthritis (Zhang et al., 2018). Likewise, it exerted an antinociceptive effect in acetic acid-induced writhing test in mice (Kwak et al., 2008).

19.6.3.2 Hipoglycemic and anti-diabetic activity

Traditional use of Gentianaceae plants containing secoiridoids for the treatment of diabetes mellitus (Anyanwu et al., 2019) has prompted an investigation of their anti-diabetic activity. Huang et al. (Huang et al., 2016) have analyzed possible anti-diabetic effects of *V. baillonii* (Gentianaceae) ethanol extract (VBFE) in HL1C hepatoma cells. Components isolated from the extract were SWS, GP, and veratriloside I−IV. Effects of VBFE and isolated components on the expression levels of phosphoenolpyruvate carboxykinase gene (Pck1) mRNA and components of insulin signaling cascade in HL1C cells were evaluated. Sweroside and GP from VBFE, as well as a mixture of these two, were able to induce phosphorylation of the insulin signal transduction pathway components—Akt S473 and ERK1/2. Further, the mixture showed the strongest inhibitory activity on the expression of Pck1, the rate-limiting enzyme in the hepatic gluconeogenesis. The described potential of SWS is very similar to insulin action. Thus this strong insulin-mimicking effect proposed SWS and GP as potential anti-diabetic agents, although the authors emphasized the necessity of additional in vivo evaluation. Indeed, a chloroform fraction (CF) of *A. vogelii* containing SWS significantly improved glucose tolerance and enhanced serum insulin levels in rats with diet- and alloxan-induced obesity-diabetes (Anyanwu et al., 2019). In vitro assays employed in this study have shown that CF significantly inhibited activities of α-amylase and α-glucosidase, enzymes involved in carbohydrates breakdown in the gut. This can be an additional hypoglycemic mechanism since inhibition of both enzymes can play an important role in diabetes management. However, the authors did not explore the contribution of single isolated compounds to the observed effect.

19.6.3.3 Antimicrobial activity

Sweroside and secoiridoid glycosides of different plant origin have also been studied for their antimicrobial effects (Horn et al., 2001; Kumarasamy, Nahar, Cox, Jaspars, & Sarker, 2003; Siler et al., 2010). Firstly, Horn et al. (Horn

et al., 2001) investigated antibacterial activity of SWS isolated from *Scabiosa columbaria* (Caprifoliaceae) against a wide range of Gram-positive (*Bacillus cereus*, *Bacillus pumulus*, *Bacillus subtilis*, *Micrococcus kristinae*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*). It has shown moderate activity with MIC of 1 mg/mL. In contrast, SWS isolated from the aerial parts of *C. erythraea* exhibited significant antibacterial activity against *B. cereus*, *B. subtilis*, *Citrobacter freundii*, *E. coli*, and *Staphylococcus epidermidis* (MIC 0.01−0.2 mg/mL) (Kumarasamy et al., 2003). A similar conclusion has been drawn by Siler et al. (Siler et al., 2010). They have demonstrated that methanol extracts of in vitro grown *C. pulchellum* and its constituents—GP, SWS, and SWM—possessed strong antibacterial and antifungal activity. Sweroside inhibited the growth of *E. coli*, *P. aeruginosa*, *S. typhimurium*, *E. cloacae*, *Listeria monocytogenes*, *B. cereus*, *Micrococcus flavus*, and *S. aureus* with MIC values of 0.01−0.02 mg/mL. It also displayed significant antifungal activity against *Aspergillus versicolor*, *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium funiculosum*, and *Trichoderma viride* (MIC values of 0.025−0.05 mg/mL). The conclusion of this research was that ethnopharmacological usage of centaury species, especially for wound-healing and anti-inflammatory treatment, could be scientifically justified with their strong antimicrobial action.

19.6.3.4 Wound-healing activity

Comprehensive investigation of the wound-healing properties of gentian-derived secoiridoids was carried out in cultured chicken embryonic fibroblasts (Oztürk, Korkmaz, Oztürk, & Başer, 2006). Methanol extract of *G. lutea* and its main components—GP, SWS, and SWM—increased the number of fibroblast cells in culture, indicating promitotic activity, stimulated cell migration and wound closure, and increased collagen production in fibroblast cells. The most active compound was SWM, as it has had the strongest effect on fibroblast cell proliferation and migration. Sweroside, on the other hand, exerted the most potent effect on the accumulation of collagen granules in fibroblast cells. However, since methanol extract was more active in all tests than pure secoiridoids, the authors have concluded that the observed effect was probably a consequence of synergistic interaction between isolated compounds.

19.6.3.5 Cytotoxic and antitumor activity

Several in vitro studies have shown the cytotoxic potential of SWS and SWS-rich extracts against human gastric carcinoma, leukemia, and pancreatic cancer cell lines (de Oliveira et al., 2013; Han, Li, Wang, Yang, & Li, 2017; Li, Di, Gao, Wang, & Zu, 2012; Wani et al., 2013). It is worth mentioning that the cytotoxic activity of SWS against human gastric carcinoma cells ($IC_{50} = 11.2\ \mu M$) was comparable to the standard anticancer drug 5-fluorouracil ($IC_{50} = 12\ \mu M$) (Li et al., 2012). Detailed analysis of the antileukemic effect of SWS was conducted using in vitro and in vivo approaches by Han et al. (2017). Sweroside dose-dependently induced S and G_2/M arrest in the HL60 promyelocytic cell line. This effect was mediated through downregulation of deregulated cell cycle checkpoints signals (cyclin D1, cyclin-dependent kinases 4, and CDC2). Concurrently, p53 and p21, important tumor suppressors, were highly induced by SWS treatment. Furthermore, SWS triggered caspase-dependent apoptosis in HL60 and in primary human leukemia cells, while no significant cytotoxic effect was observed against normal peripheral blood mononuclear cells. Most importantly, the antileukemic effect of the SWS was proven in HL-60-xenograft mouse model, where tumor growth was dose-dependently inhibited by SWS treatment (25, 50, and 100 mg/kg i.p.).

Sweroside has also shown other bioactivities emphasizing its vast pharmacological potential. Thus SWS exhibited a protective effect on cardiomyocytes against aconitine-induced toxicity in vitro (Ma et al., 2018), allergy-preventive activity in hen-egg white lysozyme sensitized mice (Oku, Ogawa, Iwaoka, & Ishiguro, 2011), neurotrophic factor-like effects in PC12h rat pheochromocytoma cells (Chiba et al., 2011), and inhibited melanogenesis of mouse melanocytes and zebrafish body pigmentation in vivo implying possible skin-whitening properties (Jeong, Jeong, Hwang, & Kim, 2015).

19.7 Conclusion

Diversity of wildlife has an enormous benefit to the different aspects of human life. It represents the foundation of human health by supporting the genetic diversity of food systems and consequently promoting health. Similarly, gaining knowledge of the anatomy, physiology, and biochemistry of distinctive representatives of flora and fauna can lead to important progress in human medicine. To the same extent, biodiversity presents a necessary resource for both traditional and modern medicine. The traditional medicine has several thousand-years-long history—Mesopotamia, Egypt, India, China, and Ancient Greece had their own lists of medicinal plants. Today, the plant-based medicine continues to

play an essential role in health care. The WHO estimated that almost 80% of the world's population in developing countries depends mainly on medicines derived from plants for their primary health system.

Besides their substantial role in traditional medicine, herbs remain the source of an enormous wealth of naturally-derived compounds, inspiring the development of modern drugs. Plant-derived compounds are evolutionarily privileged molecules with complex structures and affinities for distinctive biological targets (Appendino, Fontana, & Pollastro, 2010). Some of them possess complexity that would be nearly impossible to discover by synthetic means alone (e.g., paclitaxel), while others serve as the leads and scaffolds for the development of more efficient semisynthetic drugs. Indeed, around 70% of all new drugs introduced in the United States from 1981 to 2010 had a natural origin (Newman & Cragg, 2012). The modern drugs derived from natural sources include analgesics (salicylic acid from willow bark, morphine from papaver poppy), anti-diabetes drugs (galegine from *Galega* herb), anticancer drugs (paclitaxel from *Taxus* tree), antimalarial drugs (quinine from *Cinchona* tree), and their widely used semisynthetic counterparts (acetylsalicylic acid, metformin, docetaxel, chloroquine). The approved drugs of natural origin are the subjects of ongoing research with the aim of repurposing and/or improving existing therapies (Harhaji-Trajkovic et al., 2012; Janjetovic et al., 2011a, 2011b). Moreover, various naturally-derived compounds used in basic biomedical research provide valuable insights into molecular mechanisms underlying different phenomena (Thompson, Casali, & Chan, 2019; Zogovic et al., 2015).

In accordance with the abovementioned, xanthones and secoiridoids, plant secondary metabolites widely used in traditional medicine, possess an enormous therapeutic value. As comprehensively described in this chapter, these compounds exert potent anti-inflammatory, hepatoprotective, anti-diabetic, cardioprotective, antihypertensive, anticancer, antimicrobial, and antidepressant activity, due to myriad mechanisms of action. Some of them have been included in different clinical trials. For instance, the xanthone core is modified to obtain a simple carboxyxanthone derivative (5,6-dimethylxanthone-4-acetic acid, Vadimezan, ASA404) that reached phase III clinical trials as an antitumor agent (Ribeiro, Veloso, Fernandes, Tiritan, & Pinto, 2019). In addition, clinical trials concerning the use of the iridoid-enriched products for reducing the risk of cardiovascular diseases have already been conducted (NCT 01677169; NCT 01796561).

However, in spite of the tremendous range of biodiversity benefits to drug discovery and development, its role is still not well enough recognized and appreciated. To illustrate this ignorance, it should be highlighted that the phytochemical compositions of only 11 out of 49 protected *Gentiana*, *Gentianella, Swertia*, and *Centaurium* species are known (Tables 19.1 and 19.3). The disappearance of species, above all those that are the most sensitive/endangered, will result in the irreversible loss of an uninvestigated pool of potentially bioactive compounds. The preservation of biodiversity, as well as its sustainable use, represent not only guarantees for securing potential biotherapeutics, but overall human well-being.

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Abbreviations

5-HT Serotonin
AAPH 2,2-Azobis(2-amidinopropane)
ABTS 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACC Anterior cingulate cortex
ADMA Asymmetric dimethylarginine
ALDH-2 Aldehyde dehydrogenase
ALP Alkaline phosphatase
ALT Alanine aminotransferase
AMPA α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK AMP-activated protein kinase
ANIT α-Naphthylisothiocyanate
APAP *N*-Acetyl-p-aminophenol
AST Aspartate aminotransferase
BDNF Brain-derived neurotrophic factor
BLA Basolateral amygdala

cAMP Cyclic adenosine monophosphate
CAT Catalase
CCl_4 Carbontetrachloride
cGMP Cyclic guanosine monophosphate
COX-2 Cyclooxygenase-2
CSE Cigarette smoke extract
CSF Colony stimulating factor
CTGF Connective tissue growth factor
DA Dopamine
D-GalN D-Galactosamine
DPPH 1,1-diphenyl-2-picrylhydrazyl
DSS Dextran sulfate sodium
EC_{50} The half maximal effective concentration
ED_{50} The median effective dose
eNOS Endogenous nitric oxide synthase
ERK Extracellular signal-regulated kinase
FCA Freund's complete adjuvant
FLS Fibroblast-like synoviocytes
fMLP Formyl-L-methionyl-L-leucyl-L-phenylalanine
FRAP Ferric-reducing antioxidant power
G Guanosine
G6PC Glucose-6-phosphatase
GABA γ-Aminobutyric acid
GK Glucokinase
GM-CSF Granulocyte-macrophage colony stimulating factor
GP Gentiopicroside
GPx Glutathione peroxidase
GR Glucocorticoid receptor
GSH Glutathione
GST Glutathione-*S*-transferase
H_2O_2 Hydrogen peroxide
HDL High-density lipoproteins
HSC Hepatic stellate cells
IC_{50} The half maximal inhibitory concentration
IDO Indoleamine 2,3-double oxygen enzyme
IFN-γ Interferon-γ
IL-1 Interleukin-1
IL-10 Interleukin-10
IL-4 Interleukin-4
IL-6 Interleukin-6
InsR-α Insulin-receptor α subunit
IR Insulin resistant
IRS-1 Insulin-receptor substrate-1
IRS-2 Insulin-receptor substrate-2
JNK c-Jun N-terminal kinases
LDH Lactate dehydrogenase
LDL Low-density lipoprotein
LPL Lipoprotein lipase
LPS Lipopolysaccharide
MAO-A Monoamine oxidase A
MAO-B Monoamine oxidase B
MAPK Mitogen-activated protein kinase
MDA Malonyl dialdehyde
MIC Minimal inhibitory concentration
MMP Matrix metalloproteinase
MPO Myeloperoxidase
NAc Nucleus accumbens
NE Norepinephrine

NF-κB Nuclear factor-kappa B
NGF Nerve growth factor
NMDA *N*-methyl-D-aspartate
NO Nitric oxide
NTG Nitroglycerin
ox-LDL Oxidized low-density lipoprotein
PAF Platelet-activating factor
PDGF Platelet-derived growth factor
PEPCK Phosphoenolpyruvate carboxykinase
PGE2 Prostaglandin E2
PI Propidium iodide
PI3K Phosphatidylinositol 3-kinase
PPAR-γ Peroxisome proliferator-activated receptor-γ
PTIO 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide radical
PWL Paw withdrawal latency
RA Rheumatoid arthritis
RANKL Receptor activator of nuclear factor κB ligand
ROS Reactive oxidative species
SMA α-Smooth muscle actin
SOD Superoxide dismutase
SRBC Sheep red blood cells
SREBP-1 Sterol regulatory element-binding protein 1
STZ Streptozotocin
SWM Swertiamarin
SWS Sweroside
TG Triglyceride
TGF-β1 Transforming growth factor-β1
TIXS Total iridoids and xanthones
TNF-α Tumor necrosis α
VSMC Vascular smooth muscle cells

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Chapter 20

Grape (*Vitis vinifera* L.): health benefits and effects of growing conditions on quality parameters

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20.1 Introduction

Vine belongs to the *Vitaceae* family. Genus *Vitis* is divided into two subgenera *Muscadinia* and *Euvitis* which consist of 70 species. The most important for viticulture are the species belonging to the subgenus *Euvitis*. Furthermore, the species could be divided related to the geographical origin into North American (*Vitis labrusca*, *Vitis aestivalis*, *Vitis riparia*, *Vitis berlandieri*, *Vitis cinerea*, *Vitis rupestris*, etc.), East Asian (*Vitis amurensis*, *Vitis adnata*, etc.), and Eurasian species (*Vitis vinifera*). About 2000 cultivars of *V. vinifera* are commercially important. There are red and white cultivars, based on the presence or absences of anthocyanins in the grape berry skin, and table and wine grapes, based on their primary use.

Grapes, as the most widely grown fruit, are mostly processed into wine. Grapes are also consumed fresh or processed into raisins, juices, jams, etc. According to data from 2013, world grape production was greater than 77 million tons (Tomaz et al., 2017).

In the European Union, grape cultivation represents 45% of the wine-growing area, 60% of the total production, and 70% of the export of the world (Olalla et al., 2004).

In Mediterranean countries, *V. vinifera* L. (grapevine) products are important dietary constituents and the sources of nutraceuticals. Additionally, they have been used as medicines for the treatment of various diseases (Medicinal herbs of the regional climate, 2015; Tomaz et al., 2017).

The aim of the present chapter is to give an insight into the health benefits of grape with an emphasis on the effects of growing conditions on its quality parameters.

20.1.1 Grapevine in folk medicine

Grapevine flowers, leaves, tendrils, and grape berries which contain tannins, tartaric acid, malic acid, succinic acid and wax, sugars, and mineral substances, have been used for medicinal and therapeutic purposes.

In popular literature and folk tradition, there are available a lot of recipes for its use. Some of them are given below (Medicinal herbs of the regional climate, 2015).

Leaves used fresh or dried for preparation of tea are highly regarded as a remedy against rheumatism, gout, vomiting, and bloody sputum. Besides, the infusion from leaves is applied as a bath for cracked hands and feet due to cold. Freshly harvested and crushed leaves are placed as a coating for reducing the heat in the eyes, head, and stomach. The oil macerate of grapevine leaves (from red varieties) helps problems with circulation, enlarged veins, and cracked capillaries.

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In the weakened function of brain and spinal cord, as well as in the paralysis, the tea from grapevine flowers often achieves good success, taken as a drink or for massage. The ointment prepared from flowers is a very good remedy against sunspots.

The crushed unripe grape, used as a compress, removes heat from the eyes, head, and stomach.

The grapes are recommended to cleanse the blood and to stimulate the function of gut and kidneys.

The fresh or pasteurized grape juice not only improves appetite, and function of the digestive organs, but also aids in the release of all toxic substances. Therapy with fresh grapes or grape juice treats intestinal catarrh and chronic diarrhea. Additionally, the therapy with grape juice, applied once or twice a year, prevents the formation of gallstones, kidney stones, and bladder stones. It is strongly recommended for the inflammation of kidneys, in the weakness of glands, and in emphysema.

The very good successes are achieved in the treatment of hypertension and hypotension. In the initial state of cancer and tuberculosis, grape therapies can achieve very good results and help in the treatment with specific drugs for these diseases.

Dried raisins are a good choice for a constipation diet.

Oil from grape seed can be used as an edible oil, especially for those patients who cannot consume animal fat.

Wine can be used for the preparation of many so-called medicinal vines for various diseases: vinum aromaticum, vinum antichloroticum, vinum tartar emetics, vinum chinae, vinum diureticum, vinum chinae ferrum, vinum stomachicum etc. Many medicinal herbs and fruits can be immersed in the wine under different conditions which increase their healing properties.

Wine spirits, officially called Spiritus vini, obtained by wine distillation is used as a solvent for dissolving many medicines, and is diluted for cleansing wounds. Slightly diluted wine spirits is used for the treatment of burns. The extracts from the herbal plants in wine spirit are for internal and external use.

The wine vinegar (*Acetum vini*) cools and compresses, soothes diarrhea and bleeding when used diluted as a drink or as a compress. The compress of pure vinegar is very effective for deep sores, scars, reddening, etc. Several drops of wine vinegar, sunken in the nose, stop a bleeding nose. Diluted wine vinegar (acetic water) is used as a cure for fever reduction. Occasional washing of the body with acetic water is also suitable for a healthy person to increase the blood flow to the skin.

The precipitate (*Tartarus depuratus*) settled down in an old wine cellar is used as powder (*Tartarus emeticus*) or as wine (*Vinum stibiatum*) for vomiting.

20.2 Grape phenolics and health benefits

The oxidative deterioration of valuable biomolecules (lipids, proteins, DNA) is caused by free radicals, which are produced under the exposure to air pollutants, cigarette smoke, pesticides, toxic chemical wastes, radiation, and physical stress. The damage of biomolecules has been related with a higher risk of various diseases, for instance diabetes, gastrointestinal dysfunctions, arthritis, atherosclerosis, hemorrhagic shock, ischemia, advancing age, Alzheimer's and Parkinson's disease, carcinogenesis, and AIDS (Bagchi et al., 2000; Dimitrios, 2006). The successful antioxidant treatment should delay or prevent the onset of disease if the oxidative damage is involved in the origin of a disease (Halliwell & Whiteman, 2004; Topalović, Knežević, & Vajs, 2013).

This explains the huge volume of research work regarding the natural antioxidants, which are nowadays isolated, fully characterized, and available for various applications (Dimitrios, 2006). Antioxidants, by protecting cells against oxidative damage, prevent initiation and propagation/transformation stages of carcinogenesis (Halliwell, Gutteridge, & Cross, 1992). The possible role of the antioxidant vitamins (C and E, β-carotene), antioxidant minerals (zinc and selenium), and antioxidant enzymes (glutathione, superoxide dismutase, and catalase), have been widely evaluated in the evasion of various diseases including cancer (Halliwell et al., 1992). The treatment abilities of the abovementioned antioxidants are associated with the following features: (1) absorption and bioavailability; (2) efficient dosage, safety, and toxicity; (3) diffusion in cell compartments, organs, and extracellular fluids; (4) capacity of free radical removing; (5) ability of metal chelation; (6) gene expression activity; (7) association with enzymes involved in antioxidative defense; and (8) detoxication of carcinogenic metabolites (Bagchi et al., 2000).

The structure−activity relationship, bioavailability, and therapeutic efficacy of the synthetic and natural antioxidants differ extensively. The chemical structures of phenolic compounds determine their absorption, distribution, metabolism, and excretion (Bravo, 1998; Jimenez-Ramsey, Rogler, Housley, Butler, & Elkin, 1994).

Grapes and grape products are subject to many modern studies, especially those looking at biologically active secondary metabolites. The phenolic compounds have received great attention due to their strong anticarcinogenic, antimutagenic, antiinflammatory, antiallergenic, and antimicrobial activity (Topalović, 2012).

Phenolic compounds have in their structure one or more hydroxyl groups bound directly for one or more aromatic rings. Among three different biogenetic pathways, the shikimate/arogenate pathway (shycimic acid path) is the most important in the synthesis of phenolic compounds in plants. A key intermediate is shikimic acid, which occurs by condensation of erythrocyte-4-phosphate and phosphoenol pyruvate, over a range of intermediate products. The aromatic amino acids L-phenylalanine and L-tyrosine originate from shikimic acid. Phenylalanine is the precursor of the largest number of phenolic compounds in higher plants. In nature, phenolics are usually glycosides or esters, and rarely in the free form, but they can create complexes with some other molecules. Phenolic compounds can be divided into two groups: nonflavonoids—hydroxybenzoic and hydroxycinnamic acids and stilbenes; and flavonoids—anthocyanins, flavan-3-ols, and flavonols (Topalović, 2012).

In grapes, phenolics are mainly present in skin and seeds of berries, but small amounts are found in pulp. The flavonoids are located in skins, seeds, and stems, while nonflavonoids are in pulp. Due to the anthocyanins, the content of total phenolics is higher in the red grape varieties, in comparison to white ones. The content and composition of phenolics are affected by variety, climate, growing conditions, and ripening stage (Tomaz et al., 2017; Topalović & Mikulič-Petkovšek, 2010).

20.2.1 Biological activity of grape skin extract

The use of grape skin extract has been studied because of the potential to prevent health complications and diseases.

Jariyapamornkoon et al. determined the phytochemical content and the defensive impact of red grape skin extract (RGSE) in opposition to fructose-mediated protein oxidation (Jariyapamornkoon, Yibchok-anun, & Adisakwattana, 2013). RGSE contained total phenolics in the concentration 246.3 ± 0.9 mg gallic acid equivalent/g dried extract, total flavonoids 215.9 ± 1.3 mg catechin equivalent/g dried extract, and total anthocyanins 36.7 ± 0.8 mg cyanidin-3-glucoside equivalent/g dried extract. IC50 values of RGSE, in the DPPH radical scavenging effect was 0.03 ± 0.01 mg/mL, hydroxyl radical scavenging activity 5.40 ± 0.01 mg/mL, and superoxide radical scavenging activity 0.58 ± 0.01 mg/mL. The RGSE extensively reduced the amount of fructosamine, which is directly linked with the decrease of advanced glycation end products (AGEs) and Nε-(carboxymethyl)lysine as one of the best characterized compounds of AGEs. AGEs are the unsteady and irreversible products of the glycation procedure, which can interact with additional free amino groups leading to protein alteration (alternative protein half-life, immune system, and enzyme function), and then to physiopathological changes. RGSE prevents oxidative damage of proteins that includes influence on the thiol and protein carbonyl oxidation. RGSE exerts its valuable outcome through antioxidative and antiglycation properties for preventing AGE-mediated diabetic complication.

In a study by Hudson et al. the anticancer effect of muscadine grape skin extract (MSKE) was compared with resveratrol using primary cultures of normal prostate epithelial cells (PrEC) and cancer cell lines in different stages of prostate cancer progression (RWPE-1, WPE1-NA22, WPE1-NB14, and WPE1-NB26) (Hudson et al., 2007). The growth of all tested prostate cancer cell lines was significantly inhibited by MSKE, while the growth of normal prostate epithelial cells was not affected. Prostate cancer cell lines exhibited high rates of apoptosis in response to MSKE. In contrast to MSKE, resveratrol did not induce apoptosis through targeting PI3K−Akt and MAPK signaling pathways. Reservatrol arrested cells at the G1-S phase transition of the cell cycle was associated with increased expression of p21 and decreased expression of cyclin D1 and cyclin-dependent kinase 4 proteins. MSKE and resveratrol affect different pathways leading to inhibition of prostate cancer cell growth. Therefore MSKE might be a valuable source for further development of chemopreventive or therapeutic compounds against prostate cancer.

Obesity is a public health problem associated with a greater risk of morbidity and mortality from diabetes, heart disease, stroke, and cancers. Charradi et al. analyzed the link between obesity-induced oxidative stress, renal steatosis, and kidney dysfunction, as well as the protective effect of grape seed and skin extract (Charradi et al., 2013). The experiment was set up with rats, who were fed a usual diet or a high-fat diet for 6 weeks, treated or not treated with grape seed and skin extract. High-fat diet led to an increase in triglyceride content and kidney function disorder that are associated with an oxidative stress and reduction of copper from the kidney. The protection against fat-induced kidney lipotoxicity could be achieved by grape seed and skin extract with potential application in other kidney-related diseases, too.

Cigarette smoke is one of the main sources of oxidants in the lungs. Pires et al. implemented a dietary approach through investigation of the inflammatory cells, metalloproteinase 9 (MMP-9) activity, and oxidative stress proteins that have a part in the progress of acute lung inflammation such as superoxide dismutase, catalase, glutathione peroxidase activities, and malondialdehyde quantity (Pires et al., 2011). The hypothesis was that acute grape skin extract (GSE) usage would either decrease or prevent the acute lung inflammation induced by cigarette smoke through nitric

oxide (NO) release. This research team tested an orally active antioxidant produced from GSE in mice daily exposed to the cigarette smoke from six cigarettes for 5 days. The other group was exposed to NG-nitro-L-arginine methyl ester (an inhibitor of NO synthase) to affirm NO participation in activity of GSE. The usage of GSE prevented acute lung inflammation and oxidative damage provoked by cigarette smoke and the beneficial effects of GSE were NO-dependent. This led to reduced MMP-9 activity, a lower amount of inflammatory cells in the bronchoalveolar lavage fluid, and a decreased quantity of lipid peroxidation.

20.2.2 Biological activity of grape seed extract

There are promising results of grape seed extract for human health. The grape seed proanthocyanidin extract (GSPE) contains monomeric, dimeric, trimeric, tetrameric, and other oligomeric proanthocyanidin bioflavonoids. The proanthocyanidins soluble in water and ethanol are taken from the intestine and widely dispersed throughout all organs and fluids. Generally, the extractable proanthocyanidins (the dimer-, trimer-, and tetrameric) or bioflavonoids have been shown to be highly bioavailable and provide excellent health benefits (Jimenez-Ramsey et al., 1994). The flavonoid glycosides are more bioavailable in comparison with the pure aglycone (De Vries et al., 1998; Hollman & Katan, 1998). These low molecular proanthocyanidins, also identified as sustained release antioxidants, could persist in fluids and organs for 7−10 days and show antioxidant activity that is mechanistically diverse from other water-soluble antioxidants. However, the high-molecular-weight proanthocyanidins, being not absorbable or totally bioavailable (at all), can exhibit their antioxidant properties in the intestine and protect lipids, proteins, and carbohydrates from oxidative damage during digestion (Hagerman et al., 1998).

Bagchi et al. assessed free radical scavenging capacity of GSPE (IH636, commercially available as ActiVin) in selected in vitro and in vivo models, as well as in human clinical studies, and correlated its free radical scavenging capacity with vitamins C, E, and β-carotene (Bagchi et al., 2000).

At concentration 50 mg/L, GSPE exerted 84% and 98% greater free radical scavenging abilities against superoxide anion and hydroxyl radical, respectively, compared with vitamin E. At 100 mg/L, GSPE had 439% and 575% higher free radical scavenging abilities against superoxide anion and hydroxyl radical, respectively, in comparison with vitamin C.

Pretreatment of the 300 μg/mL tobacco-treated cells with 100 mg GSPE/mL decreased tobacco-provoked apoptosis by around 85% in oral cells. However, combined treatment of 75 μM vitamins E and C reduced apoptosis by only 46%.

In a study by Ye et al. cell growth inhibition provoked by GSPE was analyzed toward several human cancer cell lines: breast cancer cells MCF-7, gastric adenocarcinoma cells CRL 1739, and lung cancer cells A-427, and these results were compared with normal human gastric mucosal cells and murine macrophage cells J774.1 (Ye et al., 1999). At 25 and 50 mg GSPE/L showed selectivity against human cancer cell lines, while the growth and viability of the normal cells were enhanced at these GSPE concentrations.

TPA (diester of phorbol, commonly known as tetradecanoylphorbol acetate) is recognized as an activator of reactive oxygen species (ROS) and significant cancer promoter. The protective abilities of GSPE towards TPA-provoked induction of ROS in the peritoneal macrophages of mice, and lipid peroxidation and DNA fragmentation in the brain and liver of mice were analyzed in vivo. It was compared with vitamin E, vitamin C, β-carotene, and a combination of vitamins E and C. Pretreatment of mice with GSPE (25, 50, and 100 mg/kg body weight) resulted in an inhibition of TPA-induced production of ROS in peritoneal macrophage cells, and lipid peroxidation and DNA fragmentation in brain and liver tissue. GSPE, which is bioavailable to the target organs, enables considerably higher protection against free radicals than vitamins E, C, and β-carotene, as well as a combination of vitamins E and C.

In study by Pruess et al. the chronic supplementation of normotensive rats with GSPE (250 ppm) was combined with a niacin-bound chromium (5 ppm Cr) and methionine-bound zinc (18 ppm Zn) (Preuss, Motamarry, & Echard, 1997). It considerably decreased systolic blood pressure and glycosylated hemoglobin (HbA1C) and reduced lipid peroxidation and free radical production.

A study by Ray et al. analyzed the administration of nontoxic doses of GSPE in mice (3 or 7 days, 100 mg/kg, p.o.) that was followed by hepatotoxic doses of acetaminophen (400 or 500 mg/kg, i.p.) (Ray, Kumar, & Bagchi, 1998). The results showed that GSPE significantly reduced acetaminophen-provoked mortality, serum alanine aminotransferase (ALT) activity, an indicator of liver toxicity, and hepatocyte DNA damage.

In a study by Sato et al. the myocardial infarct size of Sprague-Dawley rats was decreased by 25% in a group fed with 100 mg GSPE/kg body weight for 3 weeks (Sato, Maulik, Ray, Bagchi, & Das, 1999).

The defensive impact of GSPE was found on acetaminophen-provoked nephrotoxicity and genomic DNA damage in kidneys of male ICR mice (3 months old) fed with 100 mg GSPE/kg body weight p.o. for 7 days (Ray, Patel, et al., 1998).

GSPE can produce important protection against stress-induced oxidative gastrointestinal damage through ROS scavenging. The rats were pretreated with 100 mg GSPE/kg body weight per day for 15 consecutive days and then exposed to acute and chronic stresses.

The degradation of DNA in cells induced by drug or chemicals has severe outcomes including cell death, mutation, and/or neoplastic transformation. The 7-day GSPE pretreatment before exposure to the toxicants (amiodarone, cadmium chloride, and dimethylnitrosamine—for lung, spleen, and kidney, respectively) produced almost complete protection regarding the serum chemistry changes (alanine aminotransferase, blood urea nitrogen, and creatine kinase), and prevented cell death (Ray, Hickey, & Bagchi, 1999).

A study by Banerjee et al. showed that GSPE in a dose of 100 mg three times per day provided efficient control of chronic pancreatitis symptoms (Banerjee, Trivedi, & Bagchi, 1998). It was demonstrated that GSPE could decrease the frequency of nausea and pain index in patients with chronic pancreatitis.

Bagchi et al. also reported that the administration of 1% GSPE creme 30 min prior to UVA-UVB radiation enhanced sun protection factor by 9% (Bagchi et al., 2000). Therefore topical administration of GSPE increased the sun protection factor throughout tissue conditioning and most probably by ROS scavenging.

20.3 Viticulture and studies of grape phenolic compounds in Montenegro

Grapevine is cultivated only in the southern Mediterranean part of Montenegro, in the area of the Adriatic and Adriatic-modified climate, at an altitude from 5 to 670 m above sea level. The Montenegrin grapevine growing region extends between 41°52′ and 42°42′ N and between 18°26′ and 19°29′ E (Study on the zoning of vineyard geographical production areas of Montenegro, 2017). The grapevine growing is mainly oriented to the autochthonous wine grape varieties Vranac (Fig. 20.1) and Kratošija. Vranac has undergone expansion in recent years and become the main grape variety for red wines in the neighboring countries of Montenegro (Maraš et al., 2012).

The basic characteristics of Montenegrin vineyard region are mild winters, high temperature sum, and long vegetation period. The temperature sum in the vegetation is 4785°C in the coastal region and 4467°C in Podgorica's vineyard, while the annual sum of the precipitation ranges from 1234.6 to 1937.3 mm in the coastal region and is 1659.3 mm in Podgorica's vineyard (Climatological normal, 2006). However, the distribution of rainfall is unfavorable, especially when it falls below the biologically necessary limit at critical stages of vegetation, July−August. In addition, vineyards are generally raised on easily permeable, poorly accumulating, gravel substrate. Therefore the successful production of table and wine grapes in these conditions is impossible without irrigation (Study on the zoning of vineyard geographical production areas of Montenegro, 2017).

In Montenegro, the areas under the grapevine plantation in 2017 amounted to 2850 ha, of which 2804.2 ha was productive area. The average grape yield was 7.9 t/ha, that is, 2.1 kg per vine (Statistical yearbook 2018, 2018). One of the largest vineyards in Europe, covering 2300 ha with about 11.5 million grapevines, is located at Ćemovsko Field ("Plantaže, n.d. Vineyards—The secret of Ćemovsko polje").

The studies on grapevine particularly referred to its ampelographic characteristics, the mechanical structure of the cluster, the chemical composition of must, and wine quality, especially the autochthonous variety Vranac (Pajović, Wendelin, Forneck, & Eder, 2014). The influence of different quantities and combinations of mineral fertilizers on the

FIGURE 20.1 Grape of variety Vranac at the veraison stage. *Ana Topalović, Experimental field of Biotechnical Faculty, Montenegro, 2015, personal collection.*

vegetative-productive potential of grapevine variety Vranac in conditions of skeletal soils was studied by Perović (Perović, 1991). In recent years, there has been a great interest in grape phenolic compounds and their antioxidant activities.

The polyphenolic profiles of grapes from the red grape varieties Vranac, Kratošija, and Cabernet Sauvignon grown in the Montenegrin wine region were studied by Pajović et al. (Pajović, Wendelin, et al., 2014; Pajović, Raičević, et al., 2014).

The content of total anthocyanins varied significantly from 591 mg/kg in Kratošija, through 1116 mg/kg in Cabernet Sauvignon to 1360 mg/kg in Vranac. In Vranac and Kratošija, the relative concentrations of monoglucoside, nonacylated anthocyanins were different but had the same order: Mv-3-gl > Pn-3-gl > Pt-3-gl > Dp-3-gl > Cy-3-gl. In contrast to grapes of Cabernet Sauvignon, in Montenegrin varieties, the concentration of *p*-coumarylated derivates was higher than the concentration of acetylated ones. The ratio between acetylated and coumarylated anthocyanins was 0.15 in Kratošija, 0.46 in Vranac, and 2.07 in Cabernet Sauvignon.

The relative content of caftaric/coutaric/fertaric acid was 69/21/11 for Vranac, 75/11/14 for Kratošija, and 73/20/7 for Cabernet Sauvignon. The values for *trans*-coutaric/*trans*-caftaric ratio differed significantly and were 0.236 for Vranac, 0.107 for Kratošija, and 0.213 for Cabernet Sauvignon (Pajović, Wendelin, et al., 2014).

Pajović et al. found that the average concentration of total extractable polyphenols in grape berries for the mentioned grape varieties were different during two successive years—for Cabernet Sauvignon 2705 mg (+)catechin/kg of fresh weight (FW) of grape berries and 2017 mg/kg; for Vranac 1908 and 1598 mg/kg; for Kratošija 1699 and 1097 mg/kg (Pajović, Raičević, et al., 2014).

In comparison to Vranac and Kratošija, in the Cabernet Sauvignon grapes, on average, there were excessive amounts of low-molecular-mass proanthocyanidins LMP of 2006 and 1690 mg/kg of grape FW during two successive years; high-molecular-mass proanthocyanidins HMP of 2705 and 2805 mg/kg; and anthocyanins of 1035 mg/kg in the first year. Vranac grapes had the maximum amount of anthocyanins of 1113 mg/kg in the second year and the modest amount of LMP of 1103 and 846 mg/kg for both years. Kratošija grapes showed the lowest anthocyanin amount of 456 and 517 mg/kg, while the content of LMP was related to Vranac. The proportion of LMP among skins and seeds were 34:66, 39:61, and 49:51, while the proportion of HMP were 67:33, 62:38, and 64:36 for Vranac, Kratošija, and Cabernet Sauvignon, respectively. Every one of the analyzed grape varieties demonstrated higher HMP level in the skin and higher LMP amount in the seeds.

Bogićević studied flavanol accumulation in grapevine berries varieties Vranac and Cabernet Sauvignon grown in Ćemovsko Field from ecophysiological and enological aspects (Bogićević, 2014). Early defoliation and cluster thinning increased the concentration of anthocyanins and proanthocyanidins per berry. In Cabernet Sauvignon, this effect was related to berry growth, while in Vranac it was the result of increased synthesis.

The studies with Montenegrin red wine produced from Vranac and Kratošija grape varieties were related to the effect of several enological practices on the content of catechins and proanthocyanidins (Kovač, Alonso, Bourzeix, & Revilla, 1992), the influence of yield on their total polyphenols, anthocyanins, reducing sugars, and antioxidant potential (Košmerl et al., 2013), then phenolic composition and varietal discrimination (Pajović-Šćepanović, Wendelin, & Eder, 2018; Raičević, Mijović, Popović, & Pajović-Šćepanović, 2015; Raičević et al., 2017). The red wine Vranac (from Montenegrin and Serbia wine regions) was used for the study of the antimicrobial efficiency of chosen Vranac wines toward six Gram-positive and six Gram-negative bacterial strains. When compared to the known antibiotics—chloramphenicol, streptomycin, and tetracyclin—the wines exhibited significant antibacterial activity toward Gram-positive: *Clostridum perfringens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria innocua*, *Sarcina lutea*, and . *Micrococcus flavus* and Gram-negative: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Shigella sonnei*, *Klebsiella pneumoniae*, and *Proteus vulgaris* bacteria stains (Radovanović, Jovančičević, Radovanović, & Mihajilov-Krstev, 2014).

Topalović et al. studied the changes in primary and/or secondary metabolites during the last month of ripening and at the harvest in skin, pulp, and seeds of the table grape berries of Cardinal (Topalović, Gođevac, Perović, & Trifunović, 2012; Topalović & Mikulič-Petkovšek, 2010; Topalović, Mikulič-Petkovšek, Perović, Trifunović, & Knežević, 2012; Topalović, Slatnar, Štampar, Knežević, & Veberič, 2011; Topalović, 2012).

In grape skin of Cardinal, peonidin 3-glucoside showed the highest content, next to malvidin 3-glucoside, while delphinidin 3-glucoside had the lowest concentration. Of the flavonols, quercetin 3-glucoside demonstrated the excessive content at the end of ripening, followed by quercetin glucuronide that was the main flavonol at the beginning of the last month of ripening. Of the studied flavan-3-ols, the content of (-)-epicatechin was the most abundant. On one side, the foliar fertilization of grapevine with a liquid mineral fertilizer that contained 15% P_2O_5, 20% K_2O with 0.1% B, 0.1% Mn, and 0.01% Mo (% w/w) could increase the concentration of sugars and anthocyanins, but on the other side, an effect on flavonols and flavan-3-ols was not detected. However, the climatic factors and yearly fluctuations affect the content of sugars, organic acids, and phenolic compounds (Topalović et al., 2011; Topalović, 2012).

The phenolic that showed the highest amount in grape seed was gallic acid, next to methyl gallate and monomeric flavan-3-ols. The majority of phenolics diminished during the last month of grape ripening, while the amount of some phenolics was not considerably changed. The higher amount of gallic acid was observed in grape seed of Cardinal (Topalović, Gođevac, et al., 2012).

In the leaf blade and petiole of grapevine cv. Cardinal (Topalović, Mikulič-Petkovšek, et al., 2012) the following phenolics were identified: the hydroxycinnamic acid derivatives—aftaric acid and coutaric acid, a derivative of sinapic acid and glucose, and a derivative of p-coumaric acid and hexose; flavonols—quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-glucuronide, kaempferol-3-rutinoside, kaempferol-3-galactoside, kaempferol-3-glucoside, and kaempferol-3-arabinoside; and flavan-3-ols—catechin, epicatechin, and procyanidin dimers, as well as procyanidin B1 and B2. By the sum of concentrations of phenolics, the leaf blade was richer (about five times more) compared with the petiole. Quercetin-3-glucuronide was the most abundant phenolic in the leaf blade and petiole. Catechin had a lower amount in the leaf blade compared to the petiole of grapevine Cardinal.

20.4 Influence of grapevine growing conditions on grape quality and its biological activity—our results

The trial with the autochthonous grapevine variety Vranac (9-year-old vines on rootstock Kober 5BB, distance between the rows 2.4 m and between the vines 1 m) was carried out in the vineyard of Biotechnical Faculty, University of Montenegro. The vineyard is located in Lješkopolje (Podgorica) covering 20 ha with 4000 vines per ha and the average yield is around 2.5 kg grapes per vine. In the experimental grapevine rows, different doses of fertilizers and irrigation regimes were applied.

20.4.1 Growing conditions

20.4.1.1 Soil properties

Soil of top and underlying layer (0 − 30 and 30 − 60 cm) is sandy clay loam with acid reaction (active pH: 5.82 and 5.87, potential pH: 5.21 and 5.29), medium content of humus (4.5% and 3.7%), and very high available Cu (7.25 and 6.04 ppm), high content of available Fe (21.6 and 20.5 ppm), the optimum of exchangeable Ca (157 and 169 mg/100 g), and below the optimum value for exchangeable Mg (9.5 and 8.1 mg/100 g). In the topsoil, the content of available Mn (11.5 ppm) and Zn (1.13 ppm) was medium, but the content of available P (6.6 mg P_2O_5/100 g) was low, while in the underlying layer the content of the mentioned elements was at a very low level (3.7 mg P_2O_5/100 g; 7.42 ppm Mn and 0.74 ppm Zn). The K level was very high (30.7 mg K_2O/100 g) in the topsoil layer, but optimal in the underlying layer (17.7 mg K_2O/100 g). Regarding the soil conditions, the supply of nutrients is favorable in contrast to the plantations on calcareous soil, such as in Ćemovsko Field (Topalović & Knežević, 2016).

20.4.1.2 Climatic parameters

The climatic data were recorded on an automatic weather station located inside the vineyard. As known, the climatic conditions have great impacts on yields and fruit quality. The average temperature and sum of rainfall per months for the investigated period are shown in Fig. 20.2. The sum of temperatures for the period from April (budburst) to September (harvest) was similar for 2015 and 2017 (4000.4°C and 4050.2°C, respectively), but different for 2016 (3849.8°C) when sum of temperature was lower. From May to August, the average temperature in 2016 was lower than in other seasons. Significantly higher numbers of days with maximum temperatures exceeding 35°C were observed in 2015 and 2017 (34 and 39 days, respectively) in comparison to 2016 (8 days). The average values of daily maximum temperature were especially lower in 2016 than in other seasons for the periods from May 12 to May 20 (28.7°C, 19.0°C, and 27.4°C for 2015, 2016. and 2017, respectively) and from June 1 to June 15 (32.4°C, 26.0°C, and 31.5°C).

The sum of precipitation in 2016 was 589 mm, in 2017 it was 335 mm, and in 2015 it was 233.2 mm, with different distributions of rainfall. The big difference between seasons was evident from May to August, while September 2016 and 2017 were similar and more than twice as high as in 2015. Generally, in the period from flowering to veraison, rainfall in 2016 was higher than in 2015 and 2017.

20.4.1.3 Fertilization

Soil fertilization included optimal nutrition in the variant (F100), reduced nutrition with 50% of the optimal grapevine need (F50), and increased nutrition with 50% higher than the optimal needs (F150) for nitrogen, phosphorus, and

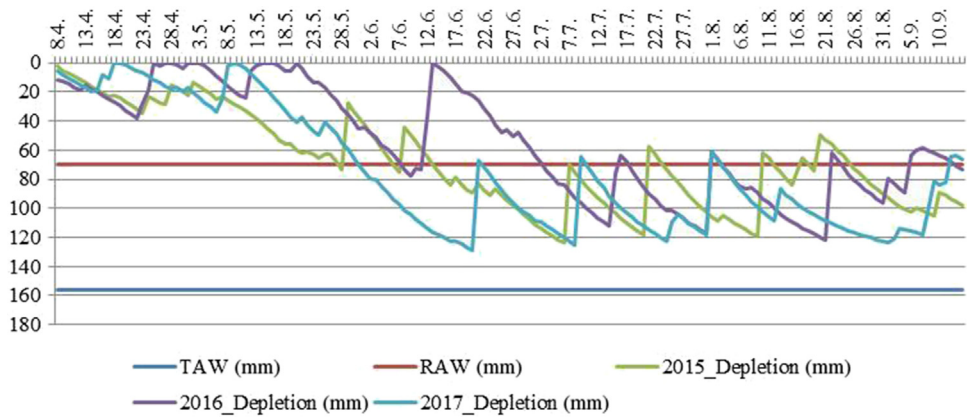

FIGURE 20.2 Sum of precipitation and average temperature per months during three successive seasons.

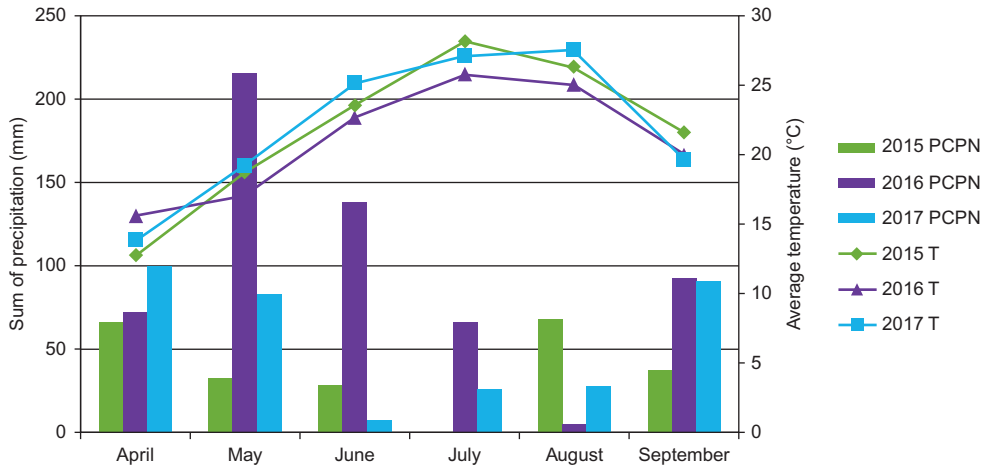

FIGURE 20.3 Soil–water depletion for reduced irrigation regime (I50) from the beginning of growing season to the harvest.

potassium. Fertilization doses for grapevine corresponded to 150, 300, and 450 kg/ha NPK 8:16:24 fertilizer for variants F50, F100, and F150, dressing with KAN (27% N) in amounts 60, 120, and 180 kg/ha.

20.4.1.4 Irrigation

Drip irrigation included optimal amount of water (conditions in which they can fully meet the grapevine need, I100), reduced amount of water (water content of the soil at the lower level of readily available water (RAW), which representing approximately 50% of the total available water (TAW) in the root zone, I50, shown in Fig. 20.3), and critical amount of water (applied in the conditions when the water content of soil was closer to the wilting point, Ic). Irrigation norms were determined based on a modified FAO Penman–Monteith method (Allen, Pereira, Raes, & Smith, 1998) by use of Aclimas, that is, EXCEL-IRR, the program for determination of the water balance in the soil and irrigation (Knežević, Životić, Perović, Topalović, & Todorović, 2017; Knežević, 2008).

During 2015 (from May 29 to August 11), total amount of water applied for vineyard irrigation was 1841 m^3/ha (through five times), 1318 m^3/ha (through five times) and 563 m^3/ha (through three times) for I100, I50, and Ic, respectively.

During 2016 (from July 11 to August 22), total amount of water applied for irrigation was 1062 m^3/ha (through three times), 598 m^3/ha (through two times) for I100 and I50, respectively. For Ic, there was no need for irrigation.

During 2017 (from June 21 to August 1), total amount of water applied for irrigation was 1542 m^3/ha (through three times), 926 m^3/ha (through three times) for I100 and I50, respectively. Similarly to 2016 for Ic, there was no need for irrigation.

The soil–water depletion for reduced irrigation regime (I50) during the experiment period (Fig. 20.3) pointed to the difference between the 2016 season and the others. Due to plenty of precipitation in mid-May and in the second half of June, the moisture content of the soil in 2016 was significantly higher.

20.4.2 Leaf and grape parameters

20.4.2.1 Elemental composition of leaf blade

According to Fregoni, as cited in Bavaresco et al. the content of nutrients in dry weight (DW) of leaf blade (Table 20.1) was adequate for optimal growth, except for K in 2015 and 2017, as well as Cu for all seasons, which were below the optimal level (Bavaresco, Gatti, & Fregoni, 2010). The chlorophyll content index (CCI) as a parameter of plant supply with nutrients was significant higher in 2016 than in the other seasons.

20.4.2.2 Elemental composition of grape skin

After conversion of the results (Table 20.2) to fresh weight by use of the average moisture content, the composition of the grape skin of variety Vranac was characterized with higher content of Ca, K, P, Mg, Mn, and lower content of Cu, Zn, and Fe (which was higher only in 2017) in comparison to the grape skin of variety Shiraz (Rogiers, Greer, Hatfield, Orchard, & Keller, 2006). Namely, 90 days after flowering in the grape skin of Shiraz the following contents were found: Ca 252 mg/kg, K 0.44%, P 320 mg/kg, Mg 130 mg/kg, Mn 2.3 mg/kg, Fe 8.0 mg/kg, Cu 2.83 mg/kg, and Zn 1.6 mg/kg.

20.4.2.3 Grape yield and total soluble solids

The high difference in yield (Table 20.3) between seasons can be attributed to fluctuations in climatic parameters and some other agricultural practices (pruning, plant protection). Popović et al. reported grape yield for the same grapevine

TABLE 20.1 The content (DW at 70°C) of elements in the leaf blade of grapevine variety Vranac (mean ± standard deviation), sampled at veraison, during three successive seasons.

Season	2015	2016	2017
L_Cu (mg/kg)	6.38 ± 0.89	5.74 ± 0.18	6.54 ± 0.20
L_Fe (mg/kg)	135.34 ± 8.42	129.41 ± 7.38	154.44 ± 10.46
L_Mn (mg/kg)	151.42 ± 16.81	134.42 ± 18.40	217.88 ± 21.44
L_Zn (mg/kg)	16.65 ± 2.64	14.41 ± 1.25	21.23 ± 1.46
L_Ca (g/kg)	39.27 ± 2.13	31.31 ± 0.81	31.66 ± 1.43
L_K (g/kg)	4.68 ± 0.52	6.43 ± 0.70	3.07 ± 0.23
L_Mg (g/kg)	5.35 ± 0.24	4.12 ± 0.29	5.50 ± 0.48
L_P (g/kg)	1.56 ± 0.15	1.53 ± 0.08	1.34 ± 0.05
L_N (%)	2.04 ± 0.07	1.94 ± 0.04	1.90 ± 0.07
CCI[a]	22.75 ± 1.33	24.40 ± 1.41	21.86 ± 1.96

[a]*CCI was measured in the vineyard.*

TABLE 20.2 The content (DW at 105°C) of elements in the skin of ripe berries of grapevine variety Vranac (mean ± standard deviation) during three successive seasons.

Season	2015	2016	2017
Cu (mg/kg)	10.37 ± 1.95	5.56 ± 0.72	14.54 ± 2.46
Fe (mg/kg)	28.01 ± 4.24	20.87 ± 4.18	59.82 ± 9.11
Mn (mg/kg)	23.84 ± 6.91	8.42 ± 2.51	31.55 ± 7.65
Zn (mg/kg)	6.44 ± 1.50	2.34 ± 0.72	6.76 ± 1.67
Ca (g/kg)	2.02 ± 0.49	1.01 ± 0.19	1.97 ± 0.43
K (g/kg)	32.23 ± 6.02	16.99 ± 3.35	17.47 ± 3.86
Mg (g/kg)	1.12 ± 0.23	0.53 ± 0.08	0.93 ± 0.17
P (g/kg)	4.49 ± 2.84	1.65 ± 0.25	2.00 ± 0.25
N (%)	1.64 ± 0.24	1.02 ± 0.16	1.82 ± 0.36

TABLE 20.3 The value of grape parameters of grapevine variety Vranac (mean ± standard deviation) measured on terrain during three successive seasons.

Season	2015	2016	2017
Yield (t/ha)	7.78 ± 2.80	7.52 ± 1.19	11.65 ± 2.09
TSS on 25-August (°Brix)	20.05 ± 1.29	18.12 ± 0.60	20.13 ± 1.24
TSS on 30-August (°Brix)	22.07 ± 1.68	20.98 ± 0.64	22.74 ± 0.67
TSS on harvest[a] (°Brix)	26.25 ± 1.88	23.22 ± 2.01	25.39 ± 1.05

[a]*Harvest was done on September 9, 2015, on September 14, 2016, and on September 5, 2017.*

variety at the same location during three successive years to be 1.13−1.52 kg/m^2. (Popović, Mijović, & Pajović, 2013). In the study of influence of yield on whole amount of polyphenols, anthocyanins, reducing sugars, and antioxidant potential in white and red wines made in Montenegrin autochthonous grape varieties (Vranac, Kratošija, Krstač, and Žižak), Košmerl et al. found that for the majority of the quality parameters the maximum value was observed at grape yield of 8 t/ha (Košmerl et al., 2013).

The amount of phostosyntate produced by the grape berry is low (10% of C required for fruit development), thus sugars imported from leaves provide starting material for most of the berry metabolism. The sugar content is important for the alcohol content and wine quality. An upper limit often indicated for the proper maturity for quality wines in warmer regions is 24 degrees Brix (Davies, Boss, Gerós, Lecourieux, & Delrot, 2012). In our experiment, the grape sugars at harvest (Table 20.3) were higher on average, because of the effect of reduced irrigation norms.

20.4.2.4 Grape total phenolics

The total phenolics were extracted from grape skin by use of ultrasound (3 h in ice) with methanol:water (80:20). Their average content ranged from 1389 mg GAE/100 g of skin FW in 2016, through 1467 mg GAE/100 g in 2015, to 1624 mg GAE/100 g in 2017.

Slinkard and Singleton found for Zinfandel 1.455 mg GAE/g of berry and for Cabernet Sauvignon 1.631 mg GAE/g, and as a mean for black grapes 1.079 mg GAE/g (Slinkard & Singleton, 1984). Katalinić et al. found for Vranac from locality Imotski (Croatia) 2252 ± 10.9 mg GAE/g (Katalinić et al., 2010). Generally, there are a lot of differences in results for the total phenolics, whose content depends on growing conditions and the method for determination, particularly for extraction.

20.4.3 Effects of growing conditions on leaf and grape parameters

The results for the 3-year experiment showed no effect of fertilization, as well as cross-interaction of fertilization and irrigation on the content of nutrients in leaf blade and on the composition of grape skin, that is, the grape quality. It could be explained by grapevine's potential to regulate take-up of nutrients from soil reserves, due to the intensive fertilization of the vineyard during previous seasons.

Further, there was a significant effect of season on all investigated parameters in leaf blade, grape skin, and grape, then the significant effect of irrigation on K, Ca, Mn, and Zn in leaf blade, as well as CCI, grape yield, and the sugars in grape berries. The cross-interaction of season and irrigation had an effect on N, Fe, and Zn in leaf blade, grape yield, and sugars.

Generally speaking, 2015 and 2017 years were more favorable than 2016, because of the suitable climatic conditions (during the period from veraison to harvest the sum of temperature was similar and higher in 2015 and 2017 than in 2016). Regarding the seasonal effect, the content of N and Ca in leaf blade and K, P and Mg in grape skin was the highest in 2015, then Fe, Mn, and Zn in leaf blade and Fe, Mn, and Cu in grape skin in 2017, while the other parameters were similar for these years. The highest amount of total soluble solids in grape was in 2015 26.25 degrees Brix and with no significant difference in comparison to 2017s 25.39 degrees Brix, while in 2016 it was significantly lower with 23.22 degrees Brix.

The grape yield was the highest in 2017, while for other seasons it was significantly lower. In 2016, the content of K in leaf blade as well as CCI was the highest in the phase of veraison.

Old research on some other grapevine varieties showed that transpiration, photosynthesis, water potential, and dry matter yields were higher at the conditions of favorable humidity (Burić, 1984). In the study on grapevine variety Cabernet Sauvignon in a 10-year-old commercial vineyard of Larissa in Greece (Koundouras et al., 2009), the total amount of applied water for the season was approximately 300 mm for the optimal irrigation treatment which was similar to our amounts.

On one hand, grapevine water restriction has a negative effect because of restricting the photosynthesis (dry matter and yield decrease), but on other hand there are several positive effects through production of abscisic acid which promotes grape ripening, restriction of competition for carbon substances by the shoot tips, and reduction of berry size. In the case of the mild water deficit, the positive effects outweigh the negative such as grapes contain less malic acid and higher level of sugars, anthocyanins, and tannins (Van Leeuwen et al., 2009).

Decreasing the irrigation norms affected negatively on the content of K and Mn in leaf blade, then chlorophyll content index and grape yield, but positively on Ca in leaf blade and total soluble solids in grape measured during the last 15 days of maturation. The content of Zn in leaf was the highest in treatment with reduced irrigation norm.

Considering the principal component analysis with varimax rotation for all seasons, the content of K and P in grape skin was in a positive relationship with N, P, and Ca in leaf blade, while the other nutrients (N, Ca, Mg, Fe, Mn, Zn, and Cu) in grape skin, as well as the grape yield, were in a positive relationship with Mg, Fe, Mn, Zn, and Cu in leaf blade but negative with K in leaf blade. Magnesium in grape skin was correlated with both groups of leaf parameters. The total soluble solids in grape berries during the last 15 days of maturation was in negative correlation with potassium in the leaf blade and chlorophyll content index (CCI) measured in time optimal for estimation of grapevine nutrient supply (veraison). It is assumed that there was more intensive transport of K from leaves to berries during the seasons with favorable climatic conditions (Martins, Cunha, Gerós, Hanana, & Blumwald, 2012), when the accumulation of sugars in berries was higher.

The results of grapevine status through the leaf and grape parameters allowed an evident separation per seasons, as illustrated by the PCA plot (Fig. 20.4). The samples of grapes from 2016 season are obviously separated from others, which are mainly explained by the first and third component. These components represent mainly elemental composition of grape (regression factor score 1) and total soluble solids during ripening and at harvest (regression factor score 3).

For all three seasons, the average content of total phenolics was 1310 mg GAE/100 g of skin FW for I100, being significantly different from 1544 mg GAE/100 g for I50, as well as 1627 mg GAE/100 g for Ic.

The clear effects exerted by water deficit on berry characteristics confirm previous studies showing the increase in the content of total phenolics in the regime of water deficiency (Fernandes et al., 2015; Koundouras et al., 2009). The increase in total polyphenol content could be caused by the fluctuations in anthocyanin content and also by the increase

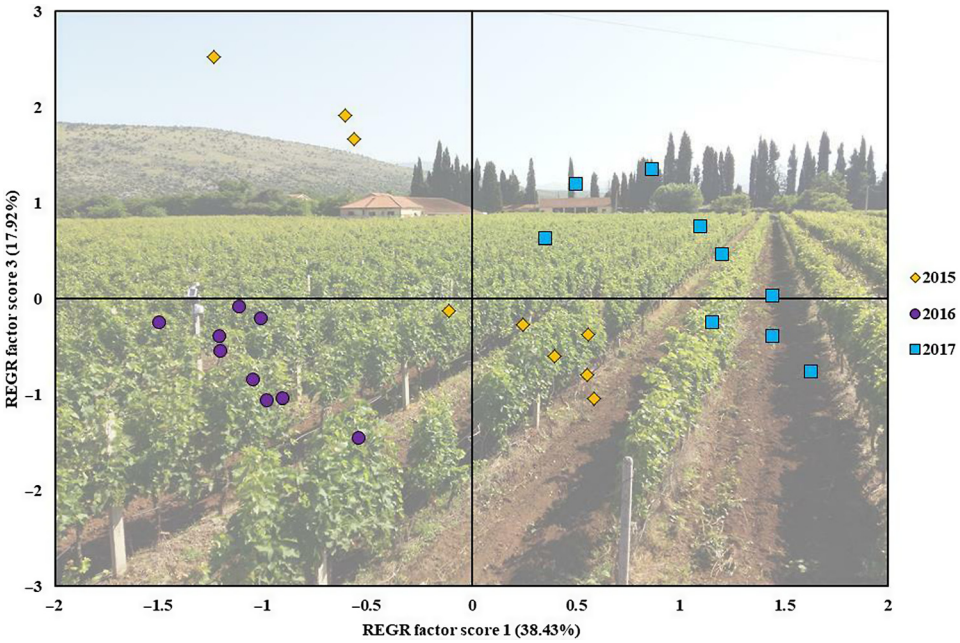

FIGURE 20.4 Principal component analysis with varimax rotation applied on the all parameters. Position of the samples along the first and third regression factor score axes. The percentages of variance explained by each axis are reported. *Background photo—Balša Bajagić, Experimental field of Biotechnical Faculty, Montenegro, 2016, personal collection.*

of other phenolic compounds. The increase in anthocyanin content is supposed to be due to the evident effect of the water deficit on increasing the sugar content. As known, the sugars have important role for the synthesis of secondary metabolites (Davies et al., 2012) and there is the positive relationship between sugar and anthocyanin concentrations (Topalović et al., 2011). Besides, the moderate and not severe water stress or drought stress has been reported to increase anthocyanin concentrations (Cheng, He, Yue, Wang, & Zhang, 2014).

There was no significant difference between seasons, although the grape from 2016 had the lowest content of total phenolics. The light/radiation and temperature represent the most influential environmental factors for the phenolic synthesis. The high light exposure can increase the concentration of phenolics and anthocyanins because of the higher activity of L-phenylalanine ammonia lyase (Teixeira, Eiras-Dias, Castellarin, & Gerós, 2013). Considering these factors, the lowest content of total phenolics in 2016 was expected.

20.4.4 Anticancer properties of grape skin extracts

One of the most prevalent cancers globally is colorectal carcinoma. It is the third most common cancer and the fourth most common cause of cancer-related death in adult populations (Stewart & Wild, 2014). The main risk factors for colorectal cancer are age, development of inflammatory bowel disease, and Crohn's disease. Other risk factors are related to lifestyle, such as unhealthy diet and physical activity habits (Mármol, Sánchez-de-Diego, Pradilla Dieste, Cerrada, & Rodriguez Yoldi, 2017). Therefore modifications in nutrition significantly reduce the possibility of developing colorectal carcinoma. For instance, polyphenols derived from grapes and turmeric exhibit chemopreventive and treatment properties toward colorectal carcinoma (Bar-Sela, Epelbaum, & Schaffer, 2010; Valenzuela et al., 2018).

The anticancer properties of grape skin extracts harvested during 2015, 2016, and 2017 in the vineyard of Biotechnical Faculty, were evaluated in colorectal carcinoma cell lines SW480 and SW620. SW480 cells are derived from a primary tumor, while the SW620 cell line represents metastatic tumor of the same patient. To assess the effects of grape skin extracts on cell viability, SW480 and SW620 cells were exposed to increasing concentrations of grape skin extracts, and viability was determined after 72 h using the MTT assay. Among tested extracts the lowest cell growth inhibition was obtained with those harvested in 2016 (Fig. 20.5). Our results are in agreement with a study by Kaur et al. that showed inhibition of colorectal carcinoma cell growth after treatment with grape seed extracts (Kaur, Agarwal, & Agarwal, 2009).

Although flavonoids and other dietary phenolics account for their antioxidant properties, in different intracellular milieu these compounds may exert prooxidant activities. This feature is particularly important in respect to their selectivity against cancer cells with increased antioxidant capacity. Thus the reduced forms of these compounds act as antioxidants while the oxidized forms such as phenoxyl radicals can promote prooxidant conditions that are unfavorable for the survival of cancer cells (Galati & O'brien, 2004). Therefore the potential of grape skin extracts to generate reactive oxygen species (ROS) in colorectal carcinoma cell lines were evaluated. ROS homeostasis is essential for normal physiological processes such as cell proliferation, survival, and migration (Boonstra & Post, 2004; Valko et al., 2007). Increased level of ROS may damage DNA, proteins, and lipids, thus interrupting normal cellular functions (Perry et al., 2000). SW480 and SW620 cells were treated with 500 μg/mL grape skin extracts for 24 h to analyze superoxide anion formation. The level of superoxide anion was assessed with fluorescent dye DHE by flow cytometry. The results were compared with well-known antioxidant coffee berry and prooxidant cisplatin (Fig. 20.6). The prooxidative effect of grape skin extracts was observed in both colorectal carcinoma cell lines (Fig. 20.6).

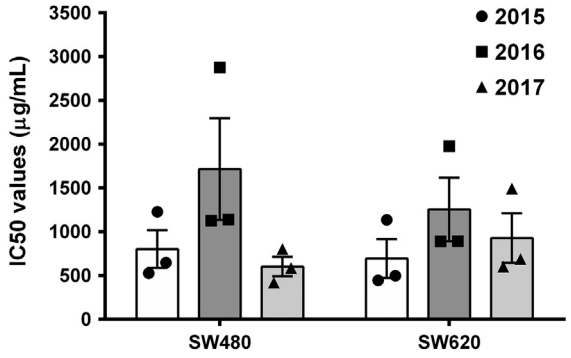

FIGURE 20.5 Cytotoxic effects of grape skin extracts from harvests 2015, 2016, and 2017 assessed in colorectal carcinoma cell lines SW480 and SW620. Following treatment with increasing concentrations of grape skin extracts from (25, 50, 250, and 500 μg/mL) the IC50 values were determined by MTT assay. IC50 value defines a concentration necessary to inhibit the cell growth by 50%. It was calculated by nonlinear regression analysis using GraphPad Prism 6.

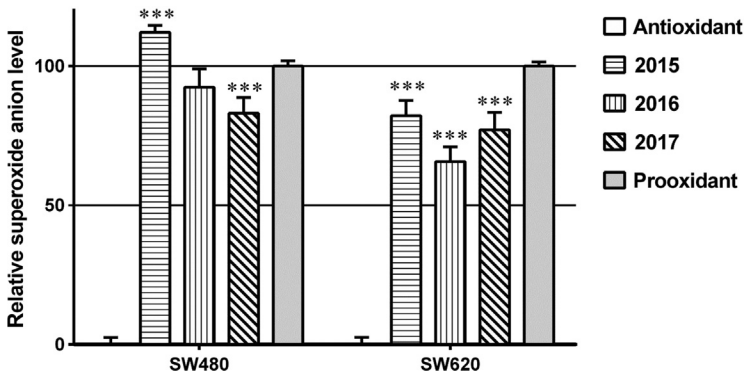

FIGURE 20.6 Superoxide anion formation provoked by grape skin extracts. DHE labeling in SW480 and SW620 cells treated with 500 μg/mL grape skin extracts for 24 h. Coffee berry was used as antioxidant and cisplatin was used as prooxidant reference compounds. Statistical significance is presented as $P < .001$ (***) and refers to cells treated with cisplatin.

Our results imply that grape skin extracts could be used as prooxidant agents in concentrations that are significantly lower (micromolar range) than concentrations typically used in other studies (millimolar range). Considering that many bioactive compounds are present in these extracts, fine tuning in concentrations and extracts' content should distinguish between extracts' antioxidant (chemoprotective) and prooxidant (cytotoxic) behavior. In order to elucidate the specific mechanisms behind this dual role, further studies are necessary.

20.4.5 Intake of elements and phenolics by grape

The humans need a certain daily intake of macro- and microelements. One grape cluster Vranac (weighing 223.61 − 236.14 g), only through skin provides the highest percent of RDA of Cu and Mn, in comparison to the other elements (Table 20.4). This estimation is done by use of our results for the partition of skin in grape and its composition on average level for 3 years. Many websites offers useful information about nutritional value of food, one of them uses data from USDA National Nutrient Database for Standard Reference and daily values based on 155 lbs (around 70 kg) body weight (Nutritional value of food, 2019). It gives the following nutritional value for 100 g of grape raw, red or green (European type, such as Thompson seedless): 1% of daily value (DV) for Ca; 2% for Fe, Mg, and P; 4% for Mn and K; and the highest 6% of DV for Cu. For banana as a favorite food, Sulaiman et al. found that 100 g of its pulp would supply 6%−10% of the K requirement for the average adult, 4%−10% of P, 4% − 13% of Mg, and 4%−133% of Mn (Sulaiman et al., 2011).

Weseler and Bastgave a review of the worldwide daily flavan-3-ol and total flavonoid intake on the basis of recently published data (from the combination of food composition databases and dietary data of large population cohort studies) (Weseler & Bast, 2017). The apples, chocolate products, grapes, tea, legumes, and wine are identified as major food sources, and the differences in habitual intake of flavonoids (Table 20.5) could be associated with sociodemographic factors. The whole amount of bioflavonoid/proanthocyanidin in a 200 g typical portion is from 50 to 500 mg, with apples having more than 200 mg (Bravo, 1998). Topalović et al. estimated that the daily consumption of total phenolics from 12 fruits (apple, banana, cherry, grapefruit, grape, kiwifruit, lemon, orange, peach, pear, plum, and strawberry) in Montenegro was 81.7 mg GAE (Topalović et al., 2013). Considering the literature data (Pajović, Raičević, et al., 2014) about total polyphenolics, low-molecular-mass proanthocyanidins and high-molecular-mass proanthocyanidins, 200 g of grape berries Vranac contain between 320 and 382 mg (expressed as catechin equivalent), but 169 − 221 mg LMP (catechin equivalent) and 255 − 329 mg HMP (cyanidin chloride equivalent), respectively.

20.5 Conclusions and future perspectives

The seasonal fluctuations in climatic parameters have a significant influence on grape quality. This research confirmed the previous findings with other grapevine varieties that the quality parameters of grape could be managed by the irrigation, during suitable climatic conditions. The reduced irrigation significantly exerts positive effects on total soluble solids and the total phenolics. The results of biological tests with colorectal carcinoma cells lines SW480 and SW620 represent new data regarding the anticancer abilities of grape skin extracts by revealing their growth inhibitory potential and prooxidative effect in colorectal carcinoma cell lines. Additional extraction, identification, and characterization of biologically active compounds from grape skin and seed of Vranac are being performed. An understanding of the apparent responsiveness of phenolics to growing conditions may yield important insights to the larger story of grape quality with regards to health benefits.

TABLE 20.4 Role of minerals, dietary reference intakes (DRIs) for minerals, intake by one grape cluster Vranac.

Element	Role	Daily intake for females 19–30 year and 31–50 year old	Daily intake for males 19–30 year and 31–50 year old	Intake by one grape cluster	% RDA
Ca	Calcium is the most abundant mineral in the body, with around 99% found in bones and teeth. Through the intracellular signaling, Ca regulates metabolic processes. In addition, calcium is important for the nerve impulse transmission, the muscle contraction, and the blood coagulation.	**1000 mg**	**1000 mg**	9.93 mg	0.99
P	Phosphorus in calcium salts is essential for healthy bone and tooth, in phospholipids for the structure of cell membranes. Intercellular P contributes to a number of processes associated with energy metabolism.	**700 mg**	**700 mg**	15.30 mg	2.19
Mg	Magnesium is generally present in all human tissues (especially in bone) and needed for muscle and nerve function. Mg activates the enzymes concerned with the replication of DNA and the synthesis of RNA and for secretion of parathyroid hormone involved in bone metabolism.	**310 mg 320 mg**	**400 mg 420 mg**	5.07 mg	F: 1.63 F: 1.58 M: 1.27 M: 1.21
K	Potassium plays a vital role in water and electrolyte balance and the normal functioning of cells, including nerves. An increased dietary intake of potassium is in relation with a decrease in blood pressure, as it promotes loss of sodium in the urine.	4.7 g	4.7 g	138 mg	2.94
Fe	Iron is essential for the binding and transport of oxygen in body by hemoglobin (in red blood cells), and has an important role in the immune system and many enzyme reactions. Fe is required for normal energy metabolism and the metabolism of drugs and foreign substances.	**18 mg**	**8 mg**	216 μg	F: 1.20 M: 2.70
Zn	Zinc activates a large number of enzymes, and as a cofactor in the superoxide dismutase enzyme, Zn is involved in protection against oxidative processes. It is involved in the major metabolic pathways concerned with protein, lipid, carbohydrate, and energy metabolism. Zn is also essential for cell division and, i.e., for growth and tissue repair as well as normal reproductive development. Additionally, Zn is required for the functioning of the immune system, the structure and function of the skin, and wound healing.	**8 mg**	**11 mg**	29 μg	F: 0.36 M: 0.26
Cu	Copper enzymes are involved in oxide-reduction processes. Cu is needed to produce red and white blood cells also for efficient iron utilization. This microelement is important for infant growth, brain development, the immune system and for strong bones.	**900 μg**	**900 μg**	59 μg	6.58
Mn	Manganese is required for bone formation and energy metabolism. As a constituent of an antioxidant enzyme, Mn helps prevent free radical-mediated damage to cells.	1.8 mg	2.3 mg	116 μg	F: 6.46 M: 5.05

Role of minerals is retrieved from British Nutrition Foundation (2018). Recommended Dietary Allowances (RDAs) in bold type and Adequate Intakes (AIs) in ordinary type. The source are DRI reports, retrieved from The National Academies of Sciences, Engineering and Medicine (2019). *F*, females; *M*, males.

TABLE 20.5 Daily flavan-3-ol and total flavonoid intake in the world (Weseler & Bast, 2017).

Geographic region	Phenolic compounds	Average daily intake mg
Europe	Flavan-3-ols	369
Ireland	Flavan-3-ols	793
Czech Republic	Flavan-3-ols	181
Mediterranean countries	Proanthocyanidins	160
Spain	Proanthocyanidins	175
Central European countries	Proanthocyanidins	114
Scandinavian countries	Proanthocyanidins	110
The Netherlands	Proanthocyanidins	96
China	Flavonoids	166
Korea	Flavonoids	105
Australia	Flavonoids	454
USA	Flavonoids	251
Brazil	Flavonoids	139

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Chapter 21

Flavonoids in cancer therapy: current and future trends

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21.1 Flavonoids

Several in vitro and in vivo studies from 20th century showed the antioxidation, antiinflammation, and anticarcinogenic effects of fruits, vegetables, and traditional plants against cancer. Based on the studies, scientist hypothesized that vegetables and fruits contain compounds that inhibit the cancer in various stages and prevent the formation of cancer. These compounds are known as "phytochemicals."

Flavonoids, as one of those compounds, are polyphenol secondary metabolites of plants and they can be extracted from almost all parts of plants (Panche, Diwan, & Chandra, 2016). Flavonoids have an important position in human dietary nutrition with daily dietary supplements reaching up to 500 mg. Approximately 10,000 flavonoids are known and several studies have supported the antioxidant, prooxidant, antiinflammatory, antiviral/bacterial, antidiabetic, cardioprotective, anticancer, and antiaging effects of flavonoids families (Wang, Li, & Bi, 2018). Most recently dietary phytochemicals have been studied and reported as preventive and can be usable against cancer by their antioxidant activities (Chikara et al., 2018).

The anticancer effects of several flavonoid families against the several types of cancer have been shown in literature. In this chapter, we reviewed the most common flavonoids families and recent in vitro and in vivo studies about the beneficial effects of common flavonoids families against several cancer types.

21.1.1 Classification and distributions of flavonoids families

Flavonoids are member of polyphenols which are naturally found in plants (Fig. 21.1). There are different classifications of flavonoid families in the literature; however, the basic skeleton of the flavonoid structure is the same. Based on the differences in their substitution and activity of these carbon skeletons, flavonoids are divided into six classes: flavones, flavonols, flavanones, flavan-3-ols, isoflavones, and anthocyanins (Joshi, Kulkarni, & Wairkar, 2018; Panche et al., 2016; Pietta, 2000; T.-Y. Wang, Li, et al., 2018). Other than those six classes; chalcones, biflavones, coumarins, and aurones are also known as flavonoids (Pietta, 2000; T.-Y. Wang, Li, et al., 2018).

21.1.1.1 Flavones

Polyphenolic flavonoids, flavones, which are luteolin, apigenin, tangeritin, baicalein, rpoifolin, diosmetin, and tricin, are present as glucosides. Members of this subgroup are generally extracted from leaves, flowers, and fruits of broccoli, green peppers, rosemary, red peppers, celery, parsley, oregano, carrot, chamomile, mint, and onion—the common names of plants which are botanically the Lamiaceae, Asteraceae, Apiaceae, Theaceae, and Rutaceae families (Hostetler, Ralston, & Schwartz, 2017; Joshi et al., 2018; Panche et al., 2016). In addition, polymethoxylated flavones are also found in citrus fruits peels.

21.1.1.2 Flavonols

The most common subgroup of flavonoids are isoflavonols which constitute proanthocyanins as building blocks. Quercetin, kaempferol, fisetin, and myricetin are found in broccoli, tomatoes, beans, onions, kale, lettuce, grapes apples,

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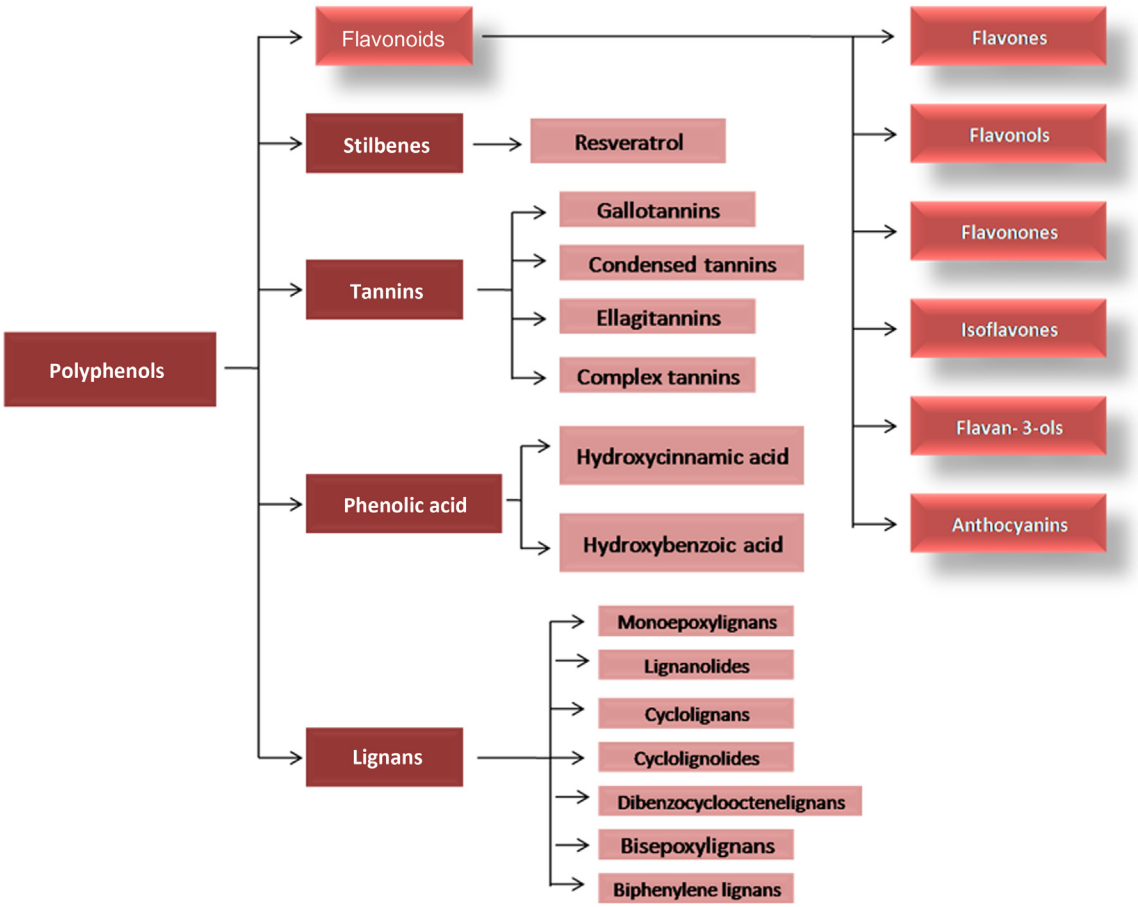


FIGURE 21.1 Flavonoids are polyphenols. *This figure is modified from Chen, L., Teng, H., Xie, Z., Cao, H., Cheang, W. S., Skalicka-Woniak, K.,... Xiao, J. (2018). Modifications of dietary flavonoids towards improved bioactivity: An update on structure-activity relationship.* Critical Reviews in Food Science and Nutrition, 58*(4), 513–527. doi:10.1080/10408398.2016.1196334.*

and berries as well as tea and red wine. In addition to their antioxidant, antiinflammatory activities, quercetin also acts as an antihistamine that can be used in hives and hayfever treatment (Joshi et al., 2018; Panche et al., 2016).

21.1.1.3 Flavanones

Hesperitin, naringenin, hesperidin, naringin, and eriodictyol are generally found in citrus fruits such as lemons, orange, and grapes. Flavanones which are also named as dihydroflavones are also important for health due to their antioxidant, antiinflammatory, blood lipid- and cholesterol-lowering activity, and cardiovascular activities. In addition, the bitter taste of citrus fruits is derived from flavanones (Joshi et al., 2018; Panche et al., 2016). Remarkable flavanones have been discovered in the last decades and the number of flavanones is being added to in the nomenclature day by day.

21.1.1.4 Flavanols, flavan-3-ols or catechins

Flavanols, flavanonols, or catechins are dihydroflavonols which are 3-hydroxy derivates of flavanones. Due to having a hydroxyl group always at position 3 of the C ring they are also known as flavan-3-ols. Flavanols are mostly found in green tea, red wine, apples, bananas, blueberries, peaches, and pears. Studies have showed that their health effect is mostly toward neurological and cardiovascular diseases (Joshi et al., 2018; Panche et al., 2016).

21.1.1.5 Isoflavones

Although distribution of isoflavones or isoflavonoids is very limited; they are mostly found in soybeans and other leguminous plants. Genistein and daidzein are known as isoflavonoids and they are mostly accepted as phytoestrogens due to the estrogenic activity of isoflavones shown in animal models (Joshi et al., 2018; Panche et al., 2016).

21.1.1.6 Anthocyanins

Anthocyanins pigments give colors to flowers and fruits in plants, and are mostly found at the outer cellular layers of berries such as blueberries, cranberries, blackberries, strawberries, raspberries, bilberries, as well as red and merlot grapes, plums, pomegranates, and red wine. Methylation or acylation of the hydroxyl groups on the A and B rings of anthocyanin results in the coloring of these fruits. Cyanidin, pelargonidin, malvidin, and delphinidin are the best known anthocyanins that show antioxidant activity, antiobesity, and antidiabetic effects, as well as cardiac related activities (Joshi et al., 2018; Panche et al., 2016).

21.1.2 Structure and biosynthesis of flavonoids

Structural and regulatory genes that are involved in biosynthesis of flavonoids have been identified in many species for many years. Although the main biosynthesis pathway of flavonoids which is called the phenylpropanoid pathway is well conserved in the plant kingdom, enzymes can be differently regulated in the pathway based on the species. For instance, hydroxylases, isomerases, reductases, and several Fe^{2+}/2-oxoglutarate-dependent dioxygenases can modify the basic flavonoid skeleton. Based on the modifications in basic skeleton of flavonoids, different subclasses are formed. The phenylpropanoid pathway starts with acetic acids and phenylalanine that converts phenylalanine into 4-coumaroyl-CoA that initiates the flavonoid biosynthesis pathway and this starting pathway is named as the shikimic acid pathway. Through chalcone synthase which is the first enzyme in flavonoid biosynthesis pathway and generates chalcones, flavonoids are derived by transferases that add sugars, methyl groups, and/or acylmoieties to the flavonoid backbone. Thus the physiological activities including reactivity, solubility, and interaction with cellular targets are modulated (Falcone Ferreyra, Rius, & Casati, 2012; T.Y. Wang, Li, et al., 2018).

Flavonoids have a common basic structure that is generally in a glycosylated or esterified form, comprised from two aromatic C6 rings that are named 7ring A and ring B, linked through an oxygen containing C3 ring that is named ring C (Fig. 21.2) (Isoda et al., 2014; Pietta, 2000; Wang et al., 2018).

Unsaturation and/or oxidation of ring C which contains different radical groups; and also the position of the link between ring B and ring C leads to formation of different flavonoid classes. When ring B is linked to ring C from the position 2 (C-2), flavones, flavonols, flavanones, anthocyanins, isoflavones, flavanols or catechins, and chalcones are formed based on the structural properties of ring C as mentioned before. This wide diversity in flavonoids comes from hydroxylation, glycosylation, methoxylation, or prenylation of the basic skeleton of flavonoids or additional radical groups (Chen, Teng, et al., 2018; T.Y. Wang, Li, et al., 2018). In addition, isoflavonoids (isoflavone, isoflavan, isoflavene, isoflavonol, isofavonone, coumestane, rotenoid, pterocarpan) are composed when ring B is linked with ring C from the position 3 (C-3) while neoflavonoids (neoflavene, arylcoumarin, etc.) are comprised from ring B linked to ring C at the position 4 (C-4). Thereby, the relationship between structure and activity of flavonoids also exhibits a wide range in nature. The structure−activity relationship (SAR) for antiinflammatory, antidiabetic, antioxidant, antiviral/bacterial, anticancer activity, and antiage-dependent neuropathology has been widely studied in the literature (Amrutha et al., 2014; Carvalho et al., 2013; Celik & Kosar, 2012; Chen, Teng, et al., 2018; Lei et al., 2012; T.Y. Wang, Li, et al., 2018).

Flavones have a unique structure by having a double bond between C2 and C3 and containing a ketone group at C4. Most flavones carry a hydroxyl group at C5 of the A ring, however, hydroxylation could also occur at position 7 of the A ring or 3′ or 4′ position of ring B (Fig. 21.3A) (Joshi et al., 2018; Panche et al., 2016). Flavones belong to the 2-benzo-γ-pyrone category of flavonoids and flavones are generally found as *O*-glycosylated at 7 position in plants as well as carrying acetyl or malonyl moieties (Hostetler et al., 2017; T.Y. Wang, Li, et al., 2018). In addition, flavones are also found as C-glycosylated at C6 and C8 which are named flavone C6- and C8-glucosides (Hostetler et al., 2017).

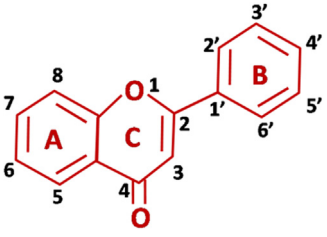
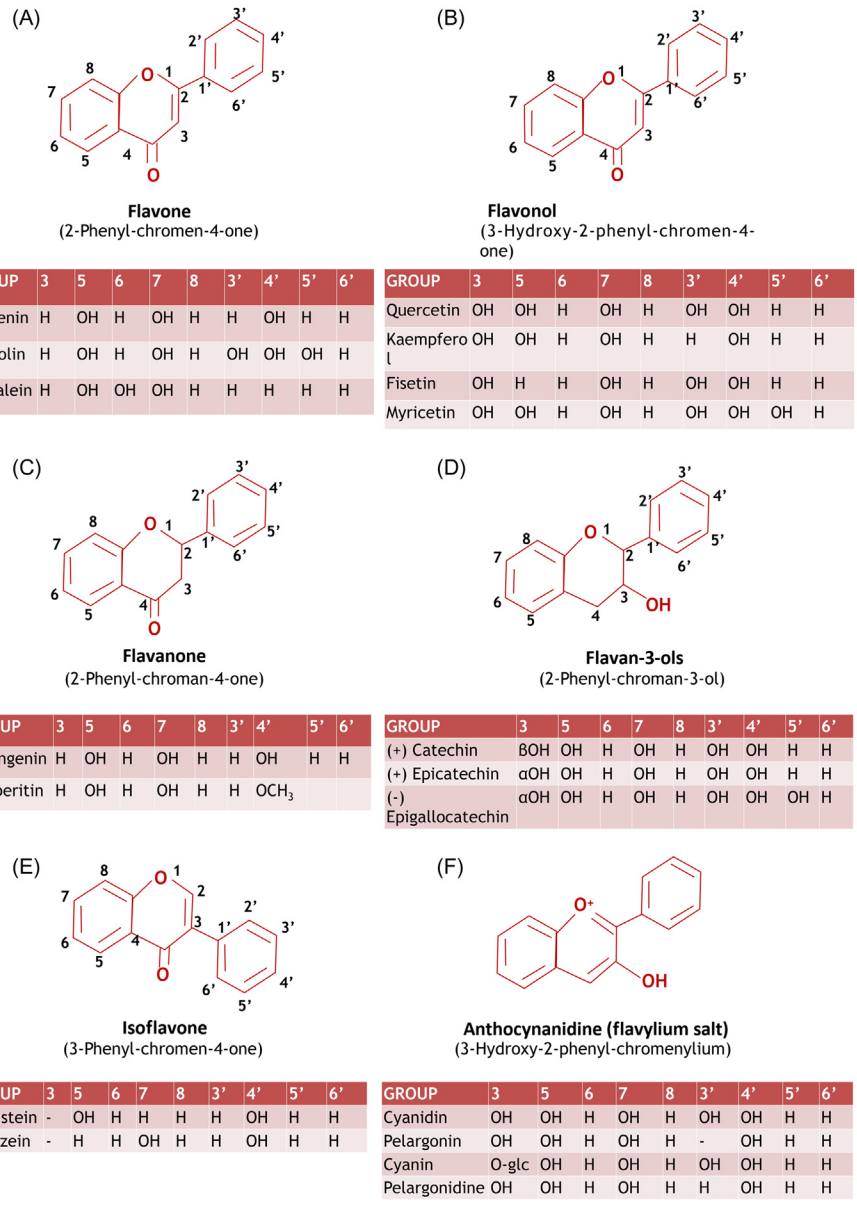


FIGURE 21.2 Basic chemical structure of flavonoids. Based on the numbering system, ring A and ring C carbons are referred to as ordinary while ring B carbons are referred to by primed numerals in flavonoids, except chalcones. *Adapted from Pietta, P. G. (2000). Flavonoids as antioxidants.* Journal of Natural Products, *63(7), 1035−1042.*



GROUP	3	5	6	7	8	3'	4'	5'	6'
Apigenin	H	OH	H	OH	H	H	OH	H	H
Luteolin	H	OH	H	OH	H	OH	OH	OH	H
Baicalein	H	OH	OH	OH	H	H	H	H	H

GROUP	3	5	6	7	8	3'	4'	5'	6'
Quercetin	OH	OH	H	OH	H	OH	OH	H	H
Kaempferol	OH	OH	H	OH	H	H	OH	H	H
Fisetin	OH	H	H	OH	H	OH	OH	H	H
Myricetin	OH	OH	H	OH	H	OH	OH	OH	H

GROUP	3	5	6	7	8	3'	4'	5'	6'
Naringenin	H	OH	H	OH	H	H	OH	H	H
Hesperitin	H	OH	H	OH	H	H	OCH_3		

GROUP	3	5	6	7	8	3'	4'	5'	6'
(+) Catechin	βOH	OH	H	OH	H	OH	OH	H	H
(+) Epicatechin	αOH	OH	H	OH	H	OH	OH	H	H
(-) Epigallocatechin	αOH	OH	H	OH	H	OH	OH	OH	H

GROUP	3	5	6	7	8	3'	4'	5'	6'
Genistein	-	OH	H	H	H	H	OH	H	H
Daidzein	-	H	H	OH	H	H	OH	H	H

GROUP	3	5	6	7	8	3'	4'	5'	6'
Cyanidin	OH	OH	H	OH	H	OH	OH	H	H
Pelargonin	OH	OH	H	OH	H	-	OH	H	H
Cyanin	O-glc	OH	H	OH	H	OH	OH	H	H
Pelargonidine	OH	OH	H	OH	H	H	OH	H	H

FIGURE 21.3 Chemical structure of flavonoid families and their members. Chemical structure of (A) flavone, (B) flavonol, (C) flavanone, (D) flavan-3-ols, (E) isoflavone, and (F) anthocynanidine. *Adapted from Pietta, P. G. (2000). Flavonoids as antioxidants.* Journal of Natural Products, *63(7), 1035–1042.*

Like flavones, flavonols are 2-benzo-γ-pyrones and have a hydroxyl group at position 3 of the C rings (Fig. 21.3B) that can be also glycosylated. Flavonols have wide range of hydroxylation and methylation patterns as well as different glycosylation patterns, just like flavones (Joshi et al., 2018; Panche et al., 2016; T.Y. Wang, Li, et al., 2018).

Flavanones and flavanonols contain a saturated double bond between positions 2 and 3 (C2 = C3) as the only difference from flavones and are generally found together with flavones and flavonols in plants (Fig. 21.3C) (Joshi et al., 2018; Panche et al., 2016; T.Y. Wang, Li, et al., 2018).

Catechins, which are dihydroflavonols, are also known as flavan-3-ols because of their hydroxyl group at 3rd position of ring C and can be converted to flavanols. In contrast to many flavonoids, catechins do not contain a double bond between positions 2 and 3 (Fig. 21.3D), while two chiral centers are found at the 2nd and 3rd position of the molecule. Catechins are a highly multisubstituted and the three main types of catechins are monomers, dimers, and polymers (Joshi et al., 2018; Panche et al., 2016; T.Y. Wang, Li, et al., 2018).

Isoflavones which are 3-phenyl-chromones are also known as phytoestrogens and are characterized by a link between the B ring and C ring at 3rd position (Fig. 21.3E) (Joshi et al., 2018; T.Y. Wang, Li, et al., 2018).

21.2 Therapeutic potential of flavonoids in cancer

For many years, therapeutic potentials of flavonoids have been investigated on several cancer types both in vitro and in vivo. In this section, we review the flavonoids that have been shown in the literature to have antiproliferative, apoptotic, antiinvasive, antimigration, antimetastatic, and antiangiogenic properties, as well as signaling pathways that are involved in these mechanisms.

21.2.1 Antiproliferative effects of flavonoids on cancer

Several studies revealed the antiproliferative and antigrowth effects of flavonoids families on several cancer types. In this part we reviewed these promising articles.

21.2.1.1 Flavones

One of the mostly studied type of flavone, luteolin, 3′,4′,5,7-tetrahydroxyflavone, is shown to suppress or inhibit cell proliferation and cell growth of several cancer types including pancreatic cancer cells (Li, Zhang, Chen, & Li, 2018), prostate cancer (Han et al., 2016), nonsmall cell lung cancer cells (Lee, Lim, et al., 2017), hepatocellular carcinoma (Yee et al., 2015) through a dose- and time-dependent manner, human placental choriocarcinoma (Lim, Yang, Bazer, & Song, 2016) through regulating miRNAs or intrinsic and/or extrinsic death signaling pathways and activating TGF-β pathway. In addition, it has been documented that luteolin inhibits the tumor growth of nonsmall cell lung cancer cells through regulation of microRNA-34a-5p (Jiang, Li, et al., 2018) and significantly inhibits tumor growth in head and neck squamous cell carcinoma (HNSCC) xenograft athymic balb/c nude mouse model through inhibition of p300 acetyltransferase (Selvi et al., 2015), respectively. Other in vivo studies also revealed that luteolin has antitumor effects against gastric cancer (Lu et al., 2015) and lung adenocarcinogesis (Kasala, Bodduluru, Barua, & Gogoi, 2016), respectively. To enhance the antiproliferative and antigrowth effect of luteolin against cancer cells and tumors, luteolin-loaded nanoparticles or liposomes have been developed, thus the half-life of luteolin inside cells could be enhanced, resulting in a very low dose of luteolin having high levels of cytotoxicity for cancer cells (Qing, Wang, Li, Lu, & Liu, 2017). On the other hand, it has been documented that luteolin sensitizes the cells to chemotherapeutic agents when used as a combination treatment. When 10 μM luteolin combined 1.7 μM doxorubicin attenuated the cytotoxicity of doxorubicin through ROS in MCF-7 breast cancer cells (Sato et al., 2015). Another study showed that luteolin enhances 5-fluorouracil effects against hepatocellular carcinoma cells through inducing apoptosis and cell cycle arrest, dose-, time-, and cell-line dependently (Xu, Yang, et al., 2016). In addition, other flavonoids, especially flavonols and flavones, can show synergetic effects with luteolin against human colorectal adenocarcinoma (Palko-Labuz, Sroda-Pomianek, Uryga, Kostrzewa-Suslow, & Michalak, 2017), cervical cancer (Lin, Hsu, et al., 2017), prostate cancer cells (Tsai et al., 2016), and breast cancer cells (Hong et al., 2018; Wilsher et al., 2017) based on their anticancer, antitumor, cytotoxic effects properties as well. Although these studies showed the effects of luteolin and flavonoids families on the same cancer cells separately, combination of the luteolin and member of other flavonoids families were not shown, while a study showed the effects of luteolin can be enhanced by flavonols in chronic lymphocytic leukemia cell lines (Sak, Kasemaa, & Everaus, 2016). A recent study showed that luteolin (5, 10, and 50 μM for in vitro and 10 mg/kg for in vivo experiments) upregulated miR-34a expression that results in a decrease in cell viability and induction of cell cycle arrest at G1 phase in gastric cancer cells and xenograft mice model. However, luteolin resistance-gastric cancer cells showed downregulated miR-34a expression level which related to poor prognosis in patients. They also found that HK1 is a direct target of miR-34 by using miRNA database and HK1 regulated luteolin effect on gastric cancer cells; and finally, when HK1 was downregulated in gastric cancer cells, luteolin resistance was partially reversed (Zhou, Ding, Lin, & Wang, 2018). Another study demonstrated the response of THP-1, HL-60, and K562 acute and chronic myeloid leukemia cells to luteolin (25−100 μM) was regulated by regulating expression of pituitary tumor-transforming gene 1 (PTTG1) oncoprotein (Chen, Tien, et al., 2018). In addition to transcriptional regulation of genes that are related to cell viability, cell survival, cell cycle, apoptosis, etc., luteolin can regulate the genes epigenetically. For instance, a study showed that 15 μM and 30 μM luteolin epigenetically inhibited activating the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant-responsive element (ARE) pathway by reducing CpG methylation of promoter region of the Nrf2 gene as well as reducing the expression and activity of DNMT1, DNMT3A, and DNMT3B DNA methyltransferases; HDAC1, HDAC2, HDAC3, HDAC6, and HDAC7 histone deacetylases (Zuo et al., 2018). Moreover, a study demonstrated that 30μM luteolin inhibited anoctamin 1 (ANO1) calcium-activated chloride channel activity as well as significantly decreased ANO-1 protein levels resulting in inhibiting the proliferation of PC-3 prostate cancer cells (Seo, Ryu, et al., 2017).

Furthermore, other studies showed that apigenin, 4′,5,7-trihydroxyflavone, inhibits cell proliferation, cell growth, and induces cell cycle arrest in several cancer cells including nasopharyngeal carcinoma cells (Zhang, Cao, et al., 2018), renal cell carcinoma (Meng, Zhu, et al., 2017), glioblastoma cells (Stump et al., 2017), human cholangiocarcinoma cells (Subhasitanont et al., 2017), bladder cancer (Shi, Shiao, Lee, & Shih, 2015), human melanoma cells (Zhao, Han, et al., 2017), colon cancer (Shan et al., 2017; Wang, Li, et al., 2017), breast cancer (Tseng et al., 2017), and pancreatic cancer (He et al., 2015) by a dose- and time-dependent fashion. Antiproliferative effect of apigenin occurs through the regulation of signaling pathways that are involved in survival and proliferation mechanisms such as P53, Wnt/β-catenin signaling, PI3K/Akt/mTOR pathway, mammalian target of rapamycin (mTOR) signaling, heat shock protein 90 (Hsp90), EGFR-mediated phosphorylation of mitogen-activated protein kinase (MAPK) pathways, and polyamine catabolism. Apigenin is inducing apoptosis by activation of P53 and inhibition of STAT3 through intracellular ROS generation in primary effusion lymphoma (PEL) (Granato et al., 2017) while enhancing cisplatin activity by P53 modulation in different cancer cells (Liu, Ji, et al., 2017). ROS generation by apigenin resulted in cellular senescence in colorectal cancer cells (Banerjee & Mandal, 2015). Antiproliferative effects of apigenin has not been only shown by in vitro studies but also in vivo studies showed the antitumor growth effect of apigenin on colon cancer and renal cell carcinoma (Ai et al., 2017; Meng, Zhu, et al., 2017). After intraperitoneal application of 30 mg/kg apigenin for every 3 day to renal cell carcinoma cell ACHN xenograft nude mice, tumor growth decreased by 50% (Meng et al., 2017). Apigenin inhibits the tumor progression of colon cancer cells through regulating STAT3-NF-κB signaling pathways together with inhibition of the colonic inflammation (Ai et al., 2017). To enhance the effect of apigenin, different drug delivery systems were used in in vitro and in vivo studies. Application of apigenin loaded liposomes decreased IC_{50} values from 43.28 to 28.22 μM and from 45.96 to 26.71 μM in HT-29 and HCT-15 cells, respectively, as well as causing a cell cycle arrest at G2/M phase suggesting that delivery method can favor the activity of apigenin in colorectal cancer cells. When apigenin-loaded micelles were used in an in vivo study, it inhibited tumor growth more efficiently compared to standard treatment with apigenin in a nude xenograft mice model of colorectal tumor (Banerjee, Banerjee, & Mandal, 2017). A similar study was repeated by using rat models and they showed that hepatocellular carcinoma development was delayed by apigenin-loaded nanoparticle application (Bhattacharya et al., 2018). Another study showed that, when HONE1 and CNE2 nasopharyngeal carcinoma cells were treated with 50 μM apigenin and 0.25 mg/mL cetuximab, apigenin enhances the antiproliferative, apoptotic effects of cetuximab on these cell lines (Hu, Liu, Zhong, & Wang, 2018). When graphene oxide-coated nanotized-apigenin was combined with paclitaxel, apigenin enhance paclitaxel activity against ovarian cancer through inducing apoptosis by ROS-dependent mitochondrial mediation (Pal et al., 2017). Although nanoparticles enhance the efficiency of flavonoids and flavonoid−drug combination against cancer cells, nanoparticles should be improved more in terms of biodegradability, drug release, and specificity of carrier to target site.

Another flavone, tangeretin, reduces or inhibits proliferation of cancer cells in a similar manner to other flavones. Studies that were performed using breast cancer cells or breast cancer rat models showed that tangeretin modulates cellular metabolic energy pathways in rats that developed 7,12-dimethylbenz(a) anthracene (DMBA)-induced proliferative breast cancer before applying tangeretin (Periyasamy, Sivabalan, Baskaran, Kasthuri, & Sakthisekaran, 2016). Additionally, it was shown in breast cancer cell lines that tangeretin regulates CYP1A1/CYP1B1-mediated metabolism that leads to an antiproliferative effect in MDA−MB−468, MCF7, and MCF10A breast cancer cells (Surichan, Arroo, Tsatsakis, & Androutsopoulos, 2018). Anticarcinogenic and antitumorigenic effect of tangeretin on 7,12-dimethylbenz (a) anthracene (DMBA)-induced proliferative breast cancer in rats was also shown by previous studies (Periyasamy et al., 2015). Anticancer properties of tangeretin that was shown in in vitro and in vivo studies can be enhanced through an emulsion-based delivery system. Based on the results of Yuwen Ting et al. on colonic carcinoma cell lines, the aqueous solubility and metabolic stability, oral-bioviability, gastric retention time, and intestinal uptake of tangeretin can be increased by using emulsion-based delivery systems (Ting, Chiou, Pan, Ho, & Huang, 2015). Thus the half-life of tangeretin or other flavonoids that have therapeutic properties can be improved during treatment in vivo.

Baicalein inhibits the proliferation and induces cell cycle arrest in cervical cancer cells through suppressing the PI3K/AKT−GSK3β pathway (Wu, Yang, Dang, Peng, & Dai, 2018) in human colorectal cancer cells (Chen, Hou, Gao, Song, & Feng, 2018), gastric cancer cells (Mu et al., 2016), and breast cancer cells with a time- and dose-dependent manner. A recent study demonstrated that 10 days treatment with different concentration of baicalein and baicalin significantly inhibited proliferation and cell growth in HCT116, HT29, and SW480 colon cancer cells but not in human foreskin fibroblast (HFF); however, baicalein was shown to be more effective than baicalin. They both also significantly increased S-phase cell cycle arrest while decreasing the G0/G1 phase in these cell lines (Dou et al., 2018). Another study demonstrated that baicalein inhibits the proliferation of DU145 and PC-3 prostate cancer cell lines (Guo et al., 2015). In addition to in vitro studies, in vivo models confirmed that baicalein inhibits tumor growth and tumor progression in gallbladder cancer

(Liu et al., 2015) and nonsmall cell lung cancer (Cathcart et al., 2016) in used xenograft mouse models. On the other hand, the roles of noncoding RNAs in several diseases including cancer have been getting more and more attention in recent years. A couple of studies showed that baicalein also regulates noncoding RNAs that can inhibit the breast cancer growth and cervical cancer progression (Yu, Cao, et al., 2018; Yu, Yang, et al., 2018). BDLNR which is a long noncoding RNA that regulates PI3K/Akt pathway is downregulated by treatment with 100 μM baicalein in HeLa and SiHa cervical cancer cell lines (Yu, Yang, et al., 2018), while another long noncoding RNA PAX8-AS1-N is upregulated by treatment with 200 μM baicalein in MDA-MB-231 and MCF-7 breast cancer cells (Yu, Cao, et al., 2018). When baicalein 50 mg/kg is combined with 10 mg/kg of docetaxel, baicalein enhances the effect of docetaxel against tumor growth in nonsmall cell lung cancer in in vivo models (Lu, Zhao, Liu, Gong, & Dong, 2018).

21.2.1.2 Flavonols

Quercetin has an antiproliferative effect on ovarian cancer cells at different concentrations and it also induces cell cycle arrest at G1 phase with 30 mg/mL treatment (Ren, Deng, Ai, Yuan, & Song, 2015). IC_{50} value of quercetin on MCF-7 breast cancer cells was detected as 37 μM and cells were arrested at G1 cell cycle phase (Ranganathan, Halagowder, & Sivasithambaram, 2015). Quercetin inhibits cell survival and proliferation of prostate cancer with a dose- and time-dependent manner while 40 μM quercetin treatment leads to maximum apoptosis rate (Ward et al., 2018). In addition to documenting the effect of quercetin against cancer progression by in vitro and in vivo studies, the targeting of several cancer types including lung cancer (Baksi et al., 2018; Zhou, Liu, Zhao, & Wang, 2018), breast cancer (Aghapour et al., 2018; de Oliveira Pedro, Goycoolea, Pereira, Schmitt, & Neumann, 2018; Lv et al., 2016; Sarkar, Ghosh, Chowdhury, Pandey, & Sil, 2016), pancreatic cancer (Serri et al., 2019), colon cancer (Xu et al., 2015), gastric cancer (Fang et al., 2018) through quercetin or its combination with chemotherapeutic drugs by using drug delivery systems was also shown in the literature. In these studies, nanoliposomes, polymeric nanoparticles, silica nanoparticles, pH-responsive amphiphilic chitosan, self-assembled micelles, and hyaluronic acid-decorated nanoparticles were used as drug delivery systems. Thus it aimed to enhance the effects of quercetin and in case of combination treatment, increasing the anticancer effects of therapeutic drugs (such as doxorubicin, gemcitabine) against cancer cells, as well as reversing drug resistance of cancer cells and reducing side effects of drugs against noncancerous cells. Some ongoing studies are aiming at improving the efficiency of quercetin only against cancer cells and not on noncancerous cells. Furthermore, scientists also focus on the encapsulation of drugs, biodegradability of carrier, sustained releasing of drug, and specificity of carrier to target site by developing new methods or carriers (Saha, Kaushik, Das, Pal, & Majumder, 2016; Wen, Zong, Hu, Li, & Wu, 2018). Not only quercetin but its derivatives also have been shown to exhibit anticancer effects on cancer cells. For instance the synthetic derivate of quercetin, 8-trifluoromethyl-3,5,7,3′,4′-*O*-pentamethyl-quercetin inhibits cell proliferation and the growth of bladder cancer cells through activation of AMPK signaling (Tao et al., 2017) while natural analog quercetin-6-C-b-D-glucopyranoside (60 μM for PC-3 and 100 μM for DU-145 cells) inhibits Akt/mTOR signaling and generation of reactive oxygen that results in an antiproliferative effect against prostate cancer cells (Hamidullah et al., 2015). Another study showed that quercetin-3′-sulfate (Q3′S) ($IC_{50} = 27.6$, for 48 h) and quercetin-3-glucuronide (Q3G) ($IC_{50} = 73.2$ μM for 48 h) inhibits growth, induces ROS-dependent apoptosis and cell cycle arrest in MCF-7 breast cancer cell lines, while quercetin also has anticancer effects on MCF-7 cell lines, having an IC_{50} of 23.1 μM (Wu, Needs, et al., 2018). When quercetin is combined with natural products such as curcumin, 2-Methoxyestradiol, PAC DP-9; natural products can increase anticancer effects of quercetin against cancer cells in triple negative breast cancer cells (Kundur et al., 2018), prostate cancer cells (Yang, Song, et al., 2015), and ovarian cancer cells (Wang, Han, et al., 2015). That is why, in addition to individual effects of quercetin and other natural products, their use as combinations can be studied for their synergistic cytotoxic effects on different cancer cells. For instance, individual or combination effects of resveratrol, quercetin, and catechin (their combination is referred as RQC) was investigated in a study, which showed that 15 μM quercetin-alone treatment is more efficient than 5 μM each of RQC at inhibition of proliferation, migration, and inducing apoptosis and cell cycle arrest in breast cancer cells. In addition, treatment of 15 or 45 mg/kg quercetin for 13 weeks (three times per week) significantly reduced tumor growth in mammary tumor xenograft female mice (Rivera, Castillo-Pichardo, Gerena, & Dharmawardhane, 2016).

A hydroxylated flavonol, fisetin, inhibits hepatic, pancreatic, and colorectal cancer cell proliferation and induces apoptosis (Youns & Abdel Halim Hegazy, 2017). Fisetin also has antiproliferative and apoptotic effects on laryngeal carcinoma through suppression of ERK1/2, inhibition of mTOR, AKT/NF-kB signaling pathways in both in vitro and in vivo (Zhang & Jia, 2016). Antiproliferative, antitumor, apoptotic effect of fisetin can be enhanced by combining with ionizing radiation or chemotherapeutic drugs. For instance, combination of fisetin (5 mg/kg per mice) and ionizing radiation (2 Gy/day for 5 days) suppressed tumor growth in xenograft tumor model of mammalian colorectal cancer

(Leu et al., 2016), whereas fisetin enhances the anticancer effects of etoposide in osteosarcoma cells (Ferreira de Oliveira et al., 2018). To enhance the antiproliferative and antitumorigenic effects of fisetin, increasing the bioavailability and enhancing efficiency of fisetin in targeting cancer cells, nanoparticles or micelles have been developed and used in in vitro and in vivo studies (Chen, Wu, et al., 2015; Feng, Yuan, et al., 2018; Kadari et al., 2017; Pawar, Singh, Rajalakshmi, Shaikh, & Bothiraja, 2018; Wang, Zhang, & Wang, 2017; Xiao et al., 2018).

Kaempferol also inhibits cell proliferation and cell growth of esophagus squamous cell carcinoma through inhibition of glycolysis and regulating EGFR signaling pathway both in vitro and in vivo (Yao et al., 2016) with a dose- and time-dependent manner. Kaempferol also has cytotoxic effects on several cancer cells including ovarian, lung, skin, pancreas, prostate, colon, breast, neuroblastoma, and glioblastoma (Pham, Sakoff, Vuong, Bowyer, & Scarlett, 2018). 50 μM kaempferol suppressed lung cancer cell proliferation through upregulating microRNA-340 as well as inducing apoptosis and autophagy via upregulated PTEN expression that led to inactivation of the PI3K/AKT pathway (Han, Liu, Gao, Zhao, & Xu, 2018). Another study showed that treatment of kaempferol (150 mg/kg/day) for 31 days on bladder cancer xenograft mice resulted in modulation of DNA methylation by downregulation of DNMT3B which is a DNA methyltransferase (Qiu et al., 2017). Besides its antiproliferative and cytotoxic effects, kaempferol can be regulated by using a mucoadhesive nanoemulsion (Colombo et al., 2018). A study showed that breast cancer cell growth was decreased by 50 μM kaempferol through estrogen in both cell lines and xenograft mice model (Kim, Hwang, & Choi, 2016). This study can be considered as a breakthrough study for the investigation of effects of flavonoids on hormones, thus one of the clinical problems, a negative effect of changing hormones level of female and male individuals during the cancer treatment, can be overcome with the stabilization of hormones to normal levels by further experiments.

21.2.1.3 Flavanones

Hesperidin from citrus seeds has a cytotoxic effect on HepG2 human hepatocellular carcinoma cell proliferation with 150.43 μM IC50 value, as well as inducing apoptosis in HepG2 through regulation of Bcl-2, Bcl-X, and caspase family of proteins (Banjerdpongchai et al., 2016). Hesperidin also decreases cell viability of A2780 human ovarian cancer cells with under 10 μM dose with a time-dependent manner through inducing GRP78, GADD153, CHOP, and cytochrome-c protein that leads to apoptosis (Zhao, Li, Gao, & De, 2017). Just as it has antiproliferative and apoptotic effects on cervical cancer cells by inducing cell cycle arrest (Wang, Yu, et al., 2015), it has similar effects on nonsmall cell lung cancer cells with dose- and time-dependent fashion (Birsu Cincin et al., 2015). In addition to in vitro assays, the anticarcinogenic effect of hesperidin on Wistar rat that was an in vivo model of renal cell carcinoma is also tested. Treatment with 100 and 200 mg/kg hesperidin for 16 weeks showed that hesperidin reverses oxidative stress, downregulates COX-2 and VEGF expressions, and inhibits the COX-2/PGE2 pathway (Siddiqi, Saidullah, & Sultana, 2018). Similar results are observed against hepatocarcinogenesis in Wistar rat through reverse oxidative stress, inflammation, PPARγ, Nrf2/ARE/HO-1, and TGF-b1/Smad3signaling (Mahmoud, Mohammed, Khadrawy, & Galaly, 2017). Moreover, hesperidin does not affect only carcinogenesis alone, but also enhances the anticarcinogenic effect of chemotherapeutic drugs which are used in clinical treatment. When etoposide was combined with hesperidin, the effect was detected to be much more than these drugs alone on cell proliferation, colony formation, cell cycle arrest, and survival of U2OS osteocarcinoma cells (Coutinho et al., 2017). Another study demonstrated that the combination of hesperidin with other natural products enhances the anticancer properties of tamoxifen against breast cancer (Khamis et al., 2018).

Naringin has antiproliferative, antigrowth effects against colorectal carcinogenesis (Zhang, Wang, Cui, & Qu, 2018) and intestinal tumorigenesis (Zhang, Li, et al., 2016).

Hesperitin, a citrus flavanone, can suppress cell proliferation, cell growth, and also tumor growth just like other members of flavanone group. Recent studies focused on enhancing the effects of hesperitin by delivering it with drug carriers such as gold nanoparticles (Gokuladhas et al., 2016), copper (II) complexes (Tamayo et al., 2016), chitosan-based nanoparticles (Mary Lazer et al., 2018), and nanocrystalline solid dispersions (NSD) (Sheokand, Navik, & Bansal, 2019). The aims of these studies are to enhance the efficiency of hesperitin, to extend the time of hesperitin in cells/tissues, to have oral bioavailability, to increase degradability of hesperetin, to target cancer cells more specifically, and to prevent the side effects of chemotherapy.

Naringenin is 4,5,7-trihydroxyflavanone, and has antiproliferative and antigrowth effects on cancer cells including colorectal cancer cells (Song et al., 2015), and placental choriocarcinoma (Park, Lim, Bazer, & Song, 2018). Combinational studies demonstrated that naringenin enhances the antiproliferative, antigrowth, and antitumor effects of chemotherapeutic drugs or inhibitors. For instance, the combination of 200 μM naringenin with 5 μM tamoxifen blocked migration and invasion as well as induced cell cycle arrest at G2/M phase and apoptosis by ROS generation in estrogen receptor positive (ER +) MCF7 breast cancer cells (Xu et al., 2018). Naringenin enhances the anticancer

effects of small molecule inhibitors of Bcl-2 ABT-737on gastric cancer cells when 40 μM naringenin is applied with 5 μM ABT-737 (Zhang, Zhong, et al., 2016). In addition to combinational therapies carrying naringenin into cells through carriers such as natural lipids-enriched chitosan nanoparticles (Kumar, Birundha, Kaveri, & Devi, 2015), copper(II) complexes (Tamayo et al., 2016), self-nanoemulsifying systems (Sandhu et al., 2017), hyaluronic acid decorated-nanoparticles (Parashar, Rathor, Dwivedi, & Saraf, 2018), biotin modified nanoparticles (Parashar, Tripathi, et al., 2018), and TPGS polymeric nanosuspension (Rajamani, Radhakrishnan, Sengodan, & Thangavelu, 2018) enhance the half-life of naringenin and activity of naringenin. In addition, carrying systems can be available for codelivery of naringenin and chemotherapeutic drugs or other flavonoids. Thus the side effects of chemotherapeutic drugs on healthy cells and tissues are avoided, as well as the activity of chemotherapeutic drugs or naringenin being enhanced. A study demonstrated that not only naringenin but also naringenin-derivates 6-C-(E-phenylethenyl) naringenin (6-CEPN), has antiproliferative, antigrowth, apoptotic, and autophagic effects on colon cancer cells (Zhao et al., 2016). A recent in vivo study, which was performed through preparation of Wistar albino rat model by using carcinogen (DMH), demonstrated that naringenin (50 mg/kg body weight, before/after applying DMH) treatment for 2 weeks has protective effects against colon cancer (Rehman et al., 2018).

21.2.1.4 Flavanols, flavan-3-ols, or catechins

A green tea polyphenol epigallocatechin gallate inhibits the proliferation and growth of breast cancer cells (Hong et al., 2017). Epigallocatechin gallate also showed chemotherapeutic potential against a cervical cancer cell line when combined with eugenol or amarogentin (Pal, Sur, Roy, Mandal, & Kumar Panda, 2018).

Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, inhibits the cell proliferation of cancer cells including soft tissue sarcoma cells (Harati et al., 2017) and esophageal cancer cells (Meng, Tong, et al., 2017). Epigallocatechin-3-gallate suppresses cell proliferation and induces apoptosis through reducing mitochondrial membrane potential and inhibiting telomerase activity in esophageal carcinoma cells (Liu, Zuo, & Wang, 2017). 5−40 μg/mL EGCG causes chromosome instability and apoptosis as well as inhibiting cell division in colon cancer cells in contrast to normal colon cells. EGCG significantly decreased chromosome instability in normal colon cells (Ni, Guo, Wang, Zhou, & Wang, 2018). In addition, EGCG inhibits proliferation and induces apoptosis in promyelocytic leukemia cells through epigenetic regulation of DNMT1, HDAC1, HDAC2, and G9a. The level of H3K9me2 was reduced while H4 histone was hyperacetylated and H3K14 histones were acetylated by EGCG (Borutinskaite, Virksaite, Gudelyte, & Navakauskiene, 2018). A next-generation sequencing study revealed that EGCG regulates novel and known microRNAs after 40 and 100 μM EGCG treatment that can be involved in MAPK signaling in nonsmall cell lung cancer cells (Bhardwaj & Mandal, 2019). A study demonstrated that ECGC inhibited cell proliferation of NB4 and HL-60 cells, induced cell cycle arrest and apoptosis through increased PCAF, C/EBPa, C/EBPE, and p27 gene expression with a dose- and time-dependent fashion, as well as 40 μM ECGC decreased DNMT1, G9A, HDAC1, and HDAC2 expression levels and histones H3 and H4 were acetylated, leading to the regulation of PCAF, C/EBPa, C/EBPE, and p27 gene promoters (Borutinskaite et al., 2018). To enhance the antiproliferative and antigrowth ability of epigallocatechin-3-gallate, honokiol nanoparticles against liver cancer (Tang et al., 2018) and RGD-containing lipid carriers against breast cancer cells (Hajipour et al., 2018) were used as well as (−)-epigallocatechin-3-gallate (EGCG)-loaded nanoparticles that were developed for targeting human MCF-7 breast cancer cell lines (Zeng, Yan, Luo, Ma, & Zhu, 2017). In addition, EGCG-encapsulated nanoparticles were used as chemotherapeutic drug carriers for prostate cancer cells (Sanna et al., 2017) and liver carcinoma cells (Liang, Chung, Gao, Yongvongsoontorn, & Kurisawa, 2018) as well as hyaluronic acid−green tea catechin micellar was used as a cisplatin carrier against ovarian cancer (Bae et al., 2017) and EGCG-nanoethosomes for docetaxel for human melanoma cells in mice (Liao et al., 2016). To enhance bioavailability of EGCG against gastric cancer tissues, fucose graft epigallocatechin-3-gallate-gold particles can be used (Yuan et al., 2018) while EGCG-loaded bovine serum albumin (BSA) encapsulated magnetite nanoparticle (MNPs) (nano EGCG) were used against lung cancer cells (Velavan, Divya, Sureshkumar, & Sudhandiran, 2018). Not only green tea polyphenols have antiproliferative, antigrowth, antitumor, apoptotic, antimigration, antiinvasion effects, but also their derivates have these abilities in both in vitro and in vivo. For instance a novel ECG analog 4-(*S*)-(2,4,6-trimethylthiobenzyl)-epigallocatechin gallate promotes ROS-dependent apoptosis and autophagy in melanoma cancer cells (Xie et al., 2017) and (−)-epigallocatechin gallate derivatives inhibit the adhesion, migration, and invasion of breast cancer cells through a decrease in the urokinase plasminogen activator and its inhibitor (Shin, Kim, Jung, & Chong, 2018) or inhibiting fatty acid synthase (Crous-Maso et al., 2018). Another study showed that epigallocatechin-3-gallate was significantly more cytotoxic and antioxidant than (+)-catechin, (+)-catechin:lysine 1:1 and (+)-catechin:lysine 1:2 in SiHa-F3 cancer cells, however, (+)-catechin:lysine 1:2 inhibits migration and melanoma metastasis in SCID mice (Payen et al., 2017).

21.2.1.5 Isoflavones

Genistein which is a member of the isoflavones, and inhibits cell proliferation through decreasing MET protein expression, increasing miR27 expression (Yang, Zang, et al., 2016), and suppressing HIF-1α, PI3K/AKT, and NF-κB/COX-2 signaling pathways in human lung cancer cells (Zhang, Su, Li, Li, & Zhao, 2017); through upregulating miR-145 expression in retinoblastoma cells (Wei, Yang, Lv, & Chen, 2017); through inhibiting STAT3 phosphorylation in pancreatic cancer cell lines (Bi, Min, Shen, & Liu, 2018); by blocking of the MAPK, hTERT expression and AKT/mTOR signaling pathways in endometrial cancer cells (Malloy et al., 2018); human cervical cancer cells through regulation of estrogen receptor-mediated PI3K/Akt-NF-κB pathway (Chen, Chen, et al., 2018); through downregulation of NF-κB and upregulation of microRNA-29b in human multiple myeloma cells (Xie, Wang, & Zhu, 2016) with a dose- and time-dependent fashion. Studies showed that genistein inhibits the proliferation and tumor growth of cancer cells through targeting DNA methylation in breast cancer in both in vitro and in vivo (Jadhav et al., 2017; Romagnolo, Donovan, Papoutsis, Doetschman, & Selmin, 2017) and in prostate cancer patient samples (Bilir et al., 2017). It was shown that isoflavones have a synergistic effect on the proliferation of breast cancer cells (Zhu, Yao, Shi, Everaert, & Ren, 2018), and the global metabolomic approach suggested that genistein, another isoflavone daidzein, and extract of soy seed can be used as chemopreventatives due to their reducing effects on glucose and glutamine uptake in breast cancer cell lines (Uifalean et al., 2016). Another metabolomics analysis demonstrated that genistein and daidzein affect the metabolites of estrogen receptors alpha and beta (ERα and ERβ) (Poschner et al., 2017). Genistein and daidzein bind to ERα and ERβ and suppress ERβ1, thus they show anticancer and/or antitumor effects on breast cancer in both in vitro and in vivo (Jiang, Fan, Cheng, Hu, & Liu, 2018). Another study showed that daidzein also regulates estrogen receptors with genistein, resulting in the inhibition of migration, invasion, and sphere formation while they promoted cell cycle arrest and apoptosis in ovarian cancer cells with dose dependently (Chan et al., 2018). That is why genistein and daidzein are also known as phytoesterogens. To enhance the cytotoxic effect of genistein against cancer cells, researchers have used drug delivery systems such as PEGylated silica hybrid nanomaterials against colon cancer cells (Pool et al., 2018), star-shaped diblock copolymer mannitol-core PLGA−TPGS against liver cancer cells (Wu, Liang, et al., 2016), and genistein-loaded chitosan nanoparticles that target folate receptors in cervical cancer cells (Cai, Yu, Hao, & Ding, 2017). In addition, a quantitative phosphoproteomic study showed that genistein modulates both DNA damage response and cell cycle pathways of triple-negative breast cancer cells (Fang et al., 2016). In addition, genistein enhances the anticancer effects of drugs, inhibitors, and other flavonoids against cancer cells through epigenetic regulation including histone acetylation (Wu, Lin, et al., 2016), with dose range between 10 μM-in both in vitro and in vivo studies.

21.2.1.6 Anthocyanins

Studies demonstrated that delphinidin which is a member of the anthocyanins group has cytotoxic and antitumor effects on cancer cells and tumor tissues including human ovarian cancer (Lim & Song, 2017), and osteosarcoma (Lee et al., 2018), as well as inducing cell cycle arrest, autophagy, and apoptosis in human leukemia (Yoshino et al., 2018; Yuan, Okusumi, et al., 2015), while delphinidin-3-glucoside represses breast carcinogenesis through inactivating the Akt/HOTAIR signaling pathway in both in vitro and in vivo (Yang, Luo, et al., 2016). When 8 μM delphinidin was administered with 5 μM arsenite, As(III), which is used in chemotherapy of acute promyelocytic leukemia (APL), delphinidin enhanced the effect of arsenite as well as induced cell cycle arrest and apoptosis (Yoshino et al., 2018). Additionally a study demonstrated that delphinidin has protective effects against the side effects of radiotherapy by protecting from radiation-induced reactive oxygen species (ROS) in normal human lung cells based on its antioxidant properties (Kim, Kim, & Kang, 2018).

21.2.2 Apoptotic effects of flavonoids on cancer

Apoptosis is a mechanism of programmed cell death which prevents cancer progression normally. However, the malfunction of cell cycle and apoptotic mechanisms lead to the occurrence of cancer cells. Studies revealed the flavonoids families have apoptotic effects on several cancer types. In this part we review the effect of several flavonoids on apoptosis in several cancer types.

21.2.2.1 Flavones

Luteolin promotes apoptosis through upregulating miR-34a (Wu, Huang, Liu, Shu, & Liu, 2015) with dose-dependent fashion and in hepatocellular cancer cells through inhibiting Akt/osteopontin signaling with a dose-dependent fashion (Im, Yeo, & Lee, 2018). Apoptosis is induced in colon cancer cells (Kang et al., 2017) and cholangiocarcinoma cells

(Kittiratphatthana, Kukongviriyapan, Prawan, & Senggunprai, 2016) through changes in mitochondrial membrane potential with ROS by 50 μg/mL and 25 μM luteolin, respectively. In addition to in vitro analysis, in vivo supportive studies were performed for glioblastoma and esophageal carcinoma cells by using 40 μM and 20, 40 μM luteolin (Chen, Zhang, et al., 2017; Wang, Wang, Jia, Pan, & Ding, 2017), respectively. Another study showed that 35 μg/mL luteolin generates ROS that lead to endoplasmic reticulum (ER) stress in melanoma cells (Kim, Kang, et al., 2016), as well as in glioblastoma (Wang, Wang, Jia, Pan, et al., 2017). Due to antioxidant activities of luteolin, 20 μg/kg of body weight of luteolin provides redox homeostasis in a liver cancer mice model (Zhang, Yang, & Wang, 2016). When 15−45 μM luteolin was applied to human leukemia cells (HL-60, U937, and MV4-11), HL-60 was highly sensitive to luteolin. 35 μM Luteolin stimulated apoptosis through Fas and FasL expression by increasing histone H3 acetylation (Wang, Chen, et al., 2018). A study showed that luteolin induces apoptosis through increasing endogenous ceramide levels and inhibiting sphingosine kinase 2 in colon cancer cells (Abdel Hadi et al., 2015).

Studies showed that apigenin induces apoptosis and cell cycle arrest in several cancer types including colon carcinoma (Wang & Zhao, 2017), glioblastoma cells (Stump et al., 2017), human cholangiocarcinoma cells (Subhasitanont et al., 2017), bladder cancer (Shi et al., 2015), human melanoma cells (Zhao et al., 2017), and human cervical cancer-derived cell lines (Souza et al., 2017) with a time- and dose-dependent manner. Yuzo Maeda et al (2018) demonstrated that apigenin has an apoptotic effect on colon cancer through suppressing Bcl-xl and Mcl-1 (Maeda et al., 2018). Apigenin induces apoptosis and autophagy by inhibiting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma cells (Yang, Pi, & Wang, 2018). Studies demonstrated that apigenin induces apoptosis through oxidative stress in breast cancer (Vrhovac Madunic et al., 2018) and human hepatocarcinoma (Kang, Molagoda, et al., 2018).

Tangeritin promotes apoptosis through cell cycle arrest and PTEN upregulation in glioma (Ma, Wang, Yu, & Zhou, 2016), and through death receptor 5 activation and inducing TRAIL-mediated apoptosis in lung cancer cells (Liu, Chen, Liu, & Zhang, 2017). In addition, proteomics analysis was performed and differently expressed proteins were identified in tangeretin-induced cell death in human AGS gastric cancer cell line (Yumnam et al., 2018). These proteins were found to be related to survival, tumor development, tumor growth, and apoptosis and they reported that KPCE regulates tangeretin-induced cell death which can be a therapeutic marker in gastric cancer treatment (Yumnam et al., 2018). Not only does tangeretin induce apoptosis in cancer cells but a derivative of tangeretin, 5-acetyloxy-6,7,8,4′- tetramethoxyflavone, also induces apoptosis in nonsmall cell lung cancer cells (NSCLC) (Li, Li, et al., 2016). G2/M arrest, autophagy, and apoptosis were observed in both CL1-5 NSCLC cell line and xenograft mice model (Li, Li, et al., 2016).

Based on the in vitro studies, baicalein has apoptotic effects on human bladder cancer cells (Choi et al., 2016) and nonsmall cell lung cancer cells (Zheng et al., 2015) with a dose- and time-dependent manner. A recent study demonstrated that treatment with different doses of baicalein and baicalin induces apoptosis through MAPK, ERK, and p38 signaling pathways and inhibits migration and colony formation in colon cancer cells but only baicalin inhibited telomerase activity that led to senescence. These in vitro observations were also confirmed with an in vivo study and they observed that baicalein and baicalin inhibited tumor growth in a colon cancer xenograft model (Dou et al., 2018).

21.2.2.2 Flavonols

In vitro studies demonstrated that quercetin induces apoptosis in several cancer types including human oral cancer (Ma, Yao, et al., 2018), breast cancer (Ranganathan et al., 2015; Seo et al., 2016), gastric cancer (Shang et al., 2018), ovarian cancer (Liu, Gong, et al., 2017), acute myeloid leukemia (Naimi et al., 2018), and colon cancer (Yang, Liu, et al., 2016) through regulating the extrinsic and/or intrinsic apoptosis signaling pathways such as the AMPK signaling pathway, Akt and Myc signaling, Twist, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) with a time- and dose-dependent fashion.

It has been documented that myricetin promotes apoptosis in several cancer cell types such as ovarian cancer cells (Xu, Xie, et al., 2016), papillary thyroid cancer cells (Ha, Jung, Kim, Bae, & Lee, 2017), hepatocellular carcinoma (Iyer, Gopal, & Halagowder, 2015; Seydi, Rasekh, Salimi, Mohsenifar, & Pourahmad, 2016), gastric cancer cells (Feng et al., 2015), and breast cancer (Jiao & Zhang, 2016) through apoptosis related events such as p53, DNA double-strand breaks, caspase-dependent, endoplasmic reticulum stress, P21 activated kinase 1 (PAK1) signaling, and BAX/BCL2-dependent pathways. It also inhibits cell proliferation and/or tumor growth and also promotes cell cycle arrest with a dose- and time-dependent manner. In a recent study, myricin was applied to two triple-negative breast cancer cell lines (MDA-MB-231, MDA-MB-468), Estrogen receptor + (ER +) MCF-7, and HER2-overexpressing breast cancer cell line and compared to normal mammary epithelial cells MCF10A; they observed that approximately 20 μM myricetin inhibited cell growth except for MCF10A cell lines. In addition, myricetin caused the accumulation of reactive oxygen species (ROS) in TNBC cells that led to mitochondria dysfunction, DNA damage, and apoptosis (Knickle, Fernando, Greenshields, Rupasinghe, & Hoskin, 2018). In addition to the cytotoxic effects of myricetin on several cancer types,

myricetin also has chemopreventive effect against tumorigenesis in intestinal tumorigenesis APC Min/ + mice and colorectal tumorigenesis Balb/c male mice models (Li, Cui, et al., 2016; Zhang et al., 2018). To enhance the cytotoxic and preventive effects of myricetin against several cancer types, scientists used the new strategies such as using drug delivery systems (Tang et al., 2016; Wang, Wang, Tang, Du, & Li, 2016), developing novel derivative of myricetin (Jose et al., 2016; Wang, Song, et al., 2018), or applying combination myricetin with other natural compounds (Huang, Chen, et al., 2015; Yi et al., 2015; Zhang, Chen, et al., 2018).

Fisetin induces apoptosis in human breast cancer cells (Guo, Zhang, Dang, Yan, & Chen, 2018; Smith, Murphy, Doucette, Greenshields, & Hoskin, 2016), nonsmall lung cancer cells (Kang, Piao, & Hyun, 2015; Kang et al., 2016), oral cancer cells (Su et al., 2017), B lymphoma cells (Lim, Lee, Byun, & Kim, 2015), renal carcinoma cells (Min, Nam, & Kwon, 2017), uveal melanoma cells (Wang, Hu, et al., 2018) through regulation of intrinsic and/or extrinsic signaling pathways including caspase family, P53 mediated-DR5 upregulation, endoplasmic stress, mitochondria-mediated signals, downregulation of Nf-kB, MAPK, and PI3K/Akt signaling pathways, and inhibition of Hsp70 expression (Kim, Lee, et al., 2015), as well as inhibiting cell proliferation and/or inducing cell cycle arrest. A study showed that treatment with 40 or 80 μM fisetin induces apoptosis and inhibits tumor growth in colorectal cancer cells even if the cells are resistant to oxaliplatin/irinotecan both in vitro and in vivo (Jeng et al., 2018). In addition to single treatment of cancer cells or mice models with fisetin, a combination of fisetin and chemotherapeutic drugs also enhances the apoptosis as well as inhibition of cell growth and cell proliferation and induces cell cycle arrest. For instance, a combination of 40 μM fisetin with 2.5 or 5 μM sorafenib and also 4 mg/kg fisetin with 10 mg/kg sorafenib activates the death receptor-5 and mitochondria-dependent apoptotic pathways in human cervical cancer cells, tissues, and xenograft mice model (Lin et al., 2016). Also when fisetin is combined with sorafenib, it enhances the apoptotic effects of sorafenib through downregulating MAPK and PI3K signaling pathways as well as inhibiting tumor growth in xenograft nude mice model of BRAF-mutant melanoma (Pal et al., 2015). Moreover, a combination of fisetin with paclitaxel induces the autophagic cell death synergetically in nonsmall cell lung cancer cells (Klimaszewska-Wisniewska et al., 2016).

Our group showed that when fisetin and hesperetin, a flavanone, were applied to HL-60 acute premyeloid leukemia cells caspase-dependent apoptosis was induced and growth of K562 was inhibited. In addition, HL-60 cells were arrested at G2/M phase by fisetin while cell cycle arrests occurred at both G0/G1 and G2/M phase by hesperetin (Adan & Baran, 2015). Similar results were also observed in K562 chronic myleoid leukemia cells. caspase-dependent apoptosis was induced, and growth of K562 was inhibited when the cells are exposed to fisetin and hesperetin (Adan & Baran, 2016).

Kaempferol is a dietary flavonoid that induces apoptosis and also cell cycle arrest in cervical cancer cells (Kashafi, Moradzadeh, Mohamadkhani, & Erfanian, 2017), ovarian cancer (Gao, Yin, Rankin, & Chen, 2018; Zhao, Tian, Wang, & Ding, 2017), bladder cancer cells (Wu, Meng et al., 2018), and lung cancer (Han, Liu, et al., 2018), as well as inducing autophagy in gastric cancer cells and lung cancer cells (Han, Liu et al., 2018) through reactive oxygen species, microRNAs, extrinsic and/or intrinsic apoptotic signaling cascades, such as P53-mediated pathway, P38, Bcl-2, Bcl-xL, death receptors, caspases, and TRAIL-induced cell death. Treatment with kaempferol and/or 5-FU decreases hTERT expression level and antiapoptotic genes (PI3K, AKT and Bcl-2) in HeLa cells (Kashafi et al., 2017). In addition, kaempferol and the combination with paclitaxel or cisplatin for 24 h promotes autophagic cell death in gastric cancer cells through regulation of the IRE1-JNK-CHOP pathway and inhibition of the HDAC/G9a axis (Kim, Lee, Kim, Cheon, & Ko, 2018). A recent study showed that 50 μM kaempferol induces endoplasmic reticulum stress-induced cell death in various cell lines through the inhibition of caspase-3 and caspase-7 (Abdullah & Ravanan, 2018).

21.2.2.3 Flavanones

Treatment with 50 μM hesperidin induces apoptosis in endometrial cancer cells through downregulating the nongenomic estrogen receptor signaling pathway (Cincin, Kiran, Baran, & Cakmakoglu, 2018). Apoptotic effects of naringenin have been documented in prostate cancer (Lim, Park, Bazer, & Song, 2017), placental choriocarcinoma (Park et al., 2018), and pancreatic cancer cells (Park, Choi, Lee, & Nam, 2017) through ROS production that encourages the signaling pathways, including P38, JNK, MAPK/ERK1/2, PI3K/AKT pathways with a dose- and time-dependent manner.

Hesperitin induces mitochondrial-pathways-mediated apoptosis through increasing ROS in esophageal cancer cells (Wu, Zhang, et al., 2016), gastric cancer cells (Zhang, Wu, et al., 2015), hepatocellular carcinoma cells (Zhang, Song, Wu, Wang, & Dong, 2015), and breast cancer cells (Palit, Kar, Sharma, & Das, 2015) with a dose- and time-dependent manner.

21.2.2.4 Flavanols, flavan-3-ols, or catechins

Epigallocatechin-3-gallate (EGCG) promotes apoptosis in human head and neck cancer (Shin et al., 2016), breast cancer (Moradzadeh, Hosseini, Erfanian, & Rezaei, 2017), cholangiocarcinoma cells (Kwak, Park, Kim, Jeong, & Kang,

2017), tongue squamous cell carcinoma (Li, Gu, et al., 2018), cervical cancer (Chakrabarty et al., 2018; Wang, Zhu, et al., 2017), and chronic myeloid leukemia cells (Xiao et al., 2019). 30 μM EGCG elevated apoptosis in head and neck cancer cells through suppression of β-catenin at mRNA and protein levels by translational regulation and proteosomal degradation in head and neck cancer cell lines and patient samples (Shin et al., 2016), while 80 μM EGCG significantly represses the telomerase activity, as well as PI3K/Akt signaling pathway that leads to apoptosis in breast cancer T47D cells (Moradzadeh et al., 2017). In addition, 100 μM EGCG induces ER stress-related apoptosis in cancer cells through regulation of PARP16 activity in cervical cancer HeLa cells and hepatocellular carcinoma QGY-7703 cells (Wang, Zhu, et al., 2017), as well as EGCG and theaflavin combination-induced apoptosis through inhibiting PI3K/Akt signaling in HeLa cells (Chakrabarty et al., 2018). Moreover, EGCG promotes apoptosis through changing mitochondrial membrane potential, caspase-independent cell death, expression and phosphorylation of Bcr/Abl gene, and also regulation of Bcr/Abl downstream pathways such as p38-MAPK/JNK and JAK2/STAT3/AKT signaling pathways in imatinib-resistance chronic myeloid leukemia (CML) cells, Bcr/Abl + CML cells, and primary CML cells (Xiao et al., 2019). In addition, EGCG induces apoptosis in B- and T-CLL primary culture cells compared to healthy cells (Cornwall, Cull, Joske, & Ghassemifar, 2016). Another study showed that exposing EGCG and epicatechin (EC) induced cell cycle arrest, ROS production, and mitochondrial-mediated apoptosis, as well as suppressed de novo fatty acid production in HEPG2 hepatocellular carcinoma cells (Khiewkamrop, Phunsomboon, Richert, Pekthong, & Srisawang, 2018). In addition, epigallocatechin gallate induces autophagy in HCT116 colon carcinoma cell line (Kim, Moon, & Park, 2016) and HEPG2 hepatocellular cancer cells (Zhao, Liu, et al., 2017), respectively.

21.2.2.5 Isoflavones

Genistein promotes apoptosis in several cancer types. For instance, genistein induces apoptosis through increased ROS level and DNA damage, upregulation of c-Jun N-terminal kinase (JNK) signal-regulating kinase 1and p38 signaling pathways in hepatocellular carcinoma (Roh, Kim, Moon, & Nam, 2016). However, genistein increased ROS production, Ca^{+2} release and changed mitochondrial membrane potential that resulted in increased apoptosis in HL-60 acute myeloid leukemia cells as well as inhibiting tumor growth in xenograft mice model (Hsiao et al., 2019). Genistein inhibited cell proliferation, induced cell cycle arrest at the G2/M phase and mitochondria-mediated apoptosis through inhibiting aerobic glycolysis in both different HCC cell lines and athymic BALB/C male mice (Li, Li, et al., 2017). 100 μM genistein upregulated microRNA-1469 that resulted in promoting apoptosis through suppressing Mcl1 gene expression in human laryngeal cancer cells (Ma et al., 2018), while genistein promotes IR-induced DNA damage, autophagy, and apoptosis through inhibition of cytoplasmic distribution of Bcl-xL and increasing nuclear Bcl-xL, leading to radiosensitivity in nonsmall cell lung cancer cells in both in vitro and in vivo (Zhang, Jin, et al., 2018). Recent study showed that when genistein was administered to mouse for 5 months, genistein suppressed development of diethylnitrosamine (DEN)-induced HCC and inhibited DEN-induced inflammation as well as genistein-induced caspase mediated-apoptosis and respiration in mitochondria of macrophages through AMPK activation through activation of AMPK pathway (Lee et al., 2019).

21.2.2.6 Anthocyanins

Delphinidin promotes apoptosis and autophagy by regulation of mTOR and AMPK signaling pathways with a dose- and time-dependent fashion in HER-2 positive breast cancer cells (Chen, Zhu, et al., 2018). Delphinidin induces TRAIL-mediated and DR5-mediated apoptosis through the activation of caspase and cleavage of histone deacetylase 3 (HDAC3) (Ko et al., 2015) or induces apoptosis by cleavage of HDAC3 downstream in prostate cancer (Jeong et al., 2016) dose- and time-dependently.

21.2.3 Antiinvasive, antimigration, and antimetastatic effects of flavonoids on cancer cell

Cancer cells have the ability to migrate and invade tissues or other organs. Based on the in vitro and in vivo studies flavonoids show tumor suppressor effects and inhibit or suppress migration, invasion, and the metastasis ability of cancer cells. In this part we reviewed the antiinvasive, antimigration, and antimetastatic effects of several flavonoids on several cancer types.

21.2.3.1 Flavones

Luteolin inhibits the migration ability of cancer cells. For instance, luteolin disrupts migration of human glioblastoma cell lines through downregulation of MMP-2/-9 and downregulation of mesenchymal markers as well as inhibition of

p-IGF-1 R/PI3K/AKT/mTOR signaling pathway (Wang, Wang, Jia, Ding, et al., 2017). Luteolin also inhibits migration in lung adenocarcinoma through proapoptotic pathways and activation of MEK/ERK signaling pathway (Meng, Chai, Li, Zhu, & Huang, 2016). In addition, luteolin deactivates STAT3 signaling and reverses IL-6-induced-MMP and EMT expression dose-dependently, thus it suppresses the invasion of pancreatic cancer PANC-1 and SW1990 cells (Huang, Dai, et al., 2015). Luteolin suppressed the migration and invasion through regulating Notch1, PI3K, AKT, mTOR, ERK, STAT3, and P38 signaling pathways as well as miRNAs in both gastric cancer cell lines and gastric cancer xenograft nude mice (Pu et al., 2018). A study showed that luteolin with oxidovanadium (IV) complex inhibits metastasis in breast cancer and colorectal cancers in both in vitro and in vivo (Naso et al., 2016). Moreover, luteolin inhibits the epithelial mesenchymal transition (EMT) through inhibiting CREB1 signaling pathways at transcriptional level in colorectal cancer cells (Liu, Lang, et al., 2017). 30 μM luteolin suppresses EMT through blocking the Notch signaling pathway in gastric cancer (Zang, Hu, Fan, et al., 2017), thus inhibiting metastasis. Luteolin reverses epithelial mesenchymal transition (EMT) through reducing β-catenin expression in both triple negative breast cancer cell lines and nude mice (Lin, Kuang, et al., 2017). Supporting results were shown in triple negative breast cancer cell lines and mice model (Cook, Liang, Besch-Williford, & Hyder, 2017). Another study showed that when luteolin is combined with cisplatin, it inhibits cell migration and invasion as well as inducing apoptosis in drug-resistant ovarian cancer through enhancing the effects of cisplatin both in vitro and in vivo (Wang, Luo, Qiao, Wu, & Huang, 2018).

Apigenin regulates some pathways which are related to migration, invasion, and metastasis. For instance, it has been demonstrated that apigenin suppresses the migration and invasion ability of colorectal cancer cell lines through inhibiting the Wnt/β-catenin signaling pathway which is crucial for the proliferation and differentiation of cells (Xu, Wang, et al., 2016). 20 μM apigenin inhibited migration, invasion, and metastasis in DLD1 and SW480 colorectal adenocarcinoma cancer cells through decreasing the expression of metastasis-related neural precursor cells and expressed developmentally downregulated 9 (NEDD9) that leads to reducing FAK, Src, and Akt phosphorylation (Dai et al., 2016). The antimigration and antiinvasion effect of apigenin on A549 lung cancer cell line was shown by Zhou, Zhongping et al. (2017), as well as the antiproliferative effect of apigenin on this cell line through regulating the Akt pathway (Zhou, Tang, et al., 2017). In addition, a recent study showed that 40 μM apigenin treatment downregulates CD26/DPPIV leading to a decreasing expression level of EGF, Snail/Slug, and Akt signaling pathways, thus inhibited colony formation, migration, invasion, and EMT in both nonsmall cell lung cancer (NSCLC) cells and in an orthotropic xenograft model (Chang et al., 2018).

Baicalein inhibits the invasion and metastatic ability of cancer cells in pancreatic cancer cells (Zhou, He, et al., 2017), nonsmall lung cancer cells (Su, Chen, & Sun, 2018), breast cancer (Ma, Yan, et al., 2016; Nguyen, Song, & Cho, 2016), ovarian cancer (Yan, Xin, et al., 2015), prostate cancer (Guo et al., 2015), and gastric cancer (Yan, Rui, & Zhang, 2015). Baicalein decreased cell viability and invasion ability of BxPC-3 (pancreatic adenocarcinoma cells) and PANC-1 (pancreatic ductal cancer cells) cells through suppressing Akt and ERK signaling (Zhou, He, et al., 2017). Studies showed that, exposing the cells to increased concentration of baicalein inhibits EMT in breast cancer cells through decreasing the expression of EMT-related genes in both in vitro and in vivo (Ma et al., 2016; Nguyen et al., 2016), which led to decreasing the invasive and metastatic abilities of the cells. Baicalein also inhibits invasion and metastasis ability of human ovarian cancer cells through inhibition of MMP-2 by suppression of MAPK-dependent NF-κB signaling pathway (Yan, Xin, et al., 2015). Another study demonstrated that treatment with baicalein inhibited metastasis in prostate cancer through regulation of caveolin-1/AKT/mTOR pathway (Guo et al., 2015). Baicalein inhibited gastric cancer cells invasion through decreasing MMP-2 and MMP-9 expression activity and also P38 signaling pathway (X. Yan et al., 2015).

21.2.3.2 Flavonols

According to in vitro and in vivo studies, quercetin has antimetastatic effects through modulating metastasis-associated factors such as GSK-3β/β-catenin and enhances the effects of anticancer drugs or ERK1/2, NF-κb, PKC-δ, and AMPKα signaling pathways as well in gastric cancer cells and mouse models (Lei, Hou, Pai, Lin, & Yeh, 2018; Li & Chen, 2018). Quercetin inhibits the migration and/or invasion ability of breast cancer cells (Tao, He, & Chen, 2015) and pancreatic cancer cells (Lee, Han, Yun, & Kim, 2015) through inhibiting EGFR expression. 30 μM quercetin suppressed invasion and migration in breast cancer cells through the suppression of glycolysis and induction of Akt/mTOR-mediated autophagy as well as suppressing glycolysis and metastasis in a xenograft mouse model (Jia et al., 2018). Another study demonstrated that exposure of 25, 50, and 100 μM quercetin inhibits TGF-β1-induced EMT in the human colorectal adenocarcinoma cell line through inhibition of Twist and increasing expression of E-cadherin (Feng, Song, et al., 2018). A study demonstrated that gold nanoparticle−conjugated quercetin significantly reduced the expression of

MMP-2, MMP-9, VEGFR-2, EMT markers such as N-cadherin, vimentin, Twist, Snail, Slug, and phosphorylation of EGFR, PI3K, GSK3β, Akt signaling pathways that lead to the inhibition of migration and invasion and capillary-like tube formation, and angiogenesis in breast cancer based on both in vitro and in vivo assays (Balakrishnan et al., 2016).

Applying 5 and 50 μM myricetin decreased invasion through promoting oxidative stress and phosphorylation of mitogen-activated protein kinase (MAPK) proteins in JAR and JEG-3 choriocarcinoma cells, respectively (Yang, Lim, Bazer, & Song, 2017). In addition, myricetin suppresses the MMP-2 and MMP-9 expression that leads to promoting the migration, invasion, and metastasis in breast cancer in both in vitro and in vivo (Ci et al., 2018).

Another member of flavonols, fisetin, also inhibits the invasion, migration, and metastasis ability of cancer cells through inhibiting MMPs and/or regulating EMT as well as targeting signaling pathways and expression of related genes. 10 and 40 μM fisetin inhibits the migration of the human lung cancer cell line through significantly downregulating the phosphorylation of ERK1/2 and MEK1 signaling (Wang & Huang, 2018). In addition, fisetin suppresses the proliferation, migration, invasion, and tumor growth through reducing EMT, VEGFR, and MAPK signaling pathways in liver cancer (Liu, Long, Miao, Liu, & Yao, 2017), while it operated via the PI3K/Akt/mTOR pathway in mammary carcinoma and breast cancer cells (Sun et al., 2018) in both cell lines and mouse models. Another study also showed fisetin reduces MMP-2 and/or MMP-9 expression, thus inhibiting the migration as well as inducing HO-1 expression (Tsai et al., 2018),and reducing invasion as well as inhibition of the PKC/ROS/MAPK pathways (Noh et al., 2015) in different breast cancer cells. A recent study showed that fisetin targets the YB-1/RSK axis in both BRAS-mutant melanoma cells and female xenograft nude mice, however, fisetin suppresses the ERK1/2 signaling pathway independently (Sechi et al., 2018). It was also documented that fisetin decreased ADAM9 expression, which is involved in glioma cancer, inhibits invasion, and enhances the phosphorylation of ERK1/2 in glioma cancer cells (Chen, Hsieh, et al., 2015). Moreover, fisetin augments the efficiency of antimetastatic and/or antiinvasive chemotherapeutic drugs such as paclitaxel in nonsmall cell lung cancer cells (Klimaszewska-Wisniewska, Halas-Wisniewska, Grzanka, & Grzanka, 2018) and sorafenib in melanoma (Pal et al., 2016). A recent study demonstrated that metastasis can inhibited by reversing EMT through suppressing the PTEN/Akt/GSK3β signaling pathway in triple negative breast cancer (TNBC) in both in vitro and in vivo (Li, Gong, et al., 2018).

The antimigration effect of kaempferol on Miapaca-2, Panc-1, and SNU-213 pancreatic cancer cells through blocking the EGFR-related signaling pathway was documented (Lee & Kim, 2016), and kaempferol inhibited the migration and invasion of liver cancer cells (Zhu et al., 2018), renal cancer cells (Hung et al., 2017), and triple-negative breast cancer cells (Li, Yan, et al., 2017). 50 μM kaempferol downregulates miR-21 that leads to downregulation of PTEN expression and inactivation of the PI3K/AKT/mTOR signaling pathway, thus inhibiting cell invasion and migration of HepG2 liver cancer cells (Zhu et al., 2018). In addition, kaempferol significantly reduces the invasion, migration through reducing MMP-2 expression, the PI3K/Akt pathway, phosphorylation of FAK in RCC 786-O renal cancer cells, and kaempferol inhibits lung metastasis in SCID mice injected with RCC 786-O cells (Hung et al., 2017). Kaempferol downregulates RhoA and Rac1 resulting in significant inhibition of migration and invasion through the reduction of EMT markers and MMP-2/-9 activity in triple negative breast cancer cells (TNBC) but not HER2-overexpressed breast cancer cells. They also demonstrated that HER2 expression recovers after the invasion and migration of kaempferol-treated TNBC cells when they transfected HER2 to TNBC cells (Li, Yan, et al., 2017). Moreover the antimetastatic effect of kaempferol has been shown in lung cancer (Jo, Park, Choi, Jeon, & Kim, 2015), breast cancer (Lee, Choi, & Hwang, 2017), and cholangiocarcinoma cell (Qin, Cui, Yang, & Tong, 2016) through regulation of EMT-related markers and/or MMPs.

21.2.3.3 Flavanones

Hesperidin inhibits migration and invasion just like flavones and flavonols. Dose- and time-dependent application of hesperidin decreased the invasion and migration ability of A549 nonsmall cell lung cancer cells through inhibiting the SDF-1/CXCR-4 signaling pathway which has a role in nonsmall cell lung cancer metastasis (Xia et al., 2018).

Naringin also inhibits migration and invasion in U251 human glioblastoma cells through inhibition of MMP-2/-9, however, cell viability was not affected under 40 μM concentration (Aroui, Najlaoui, et al., 2016). It suppresses glioblastoma cell metastasis through the inhibition of MMP-2/-9 and P38, ERK, and JNK phosphorylation (Aroui, Aouey, et al., 2016).

Naringenin inhibits the invasion and migration of gastric cancer and glioblastoma cells through inhibition of MMP-2 and MMP-9 and downregulation of Akt signaling (Bao et al., 2016; Chen, Chang, et al., 2018). A recent study demonstrated that naringenin blocks the voltage-gated sodium channels that results in blocking the metastasizing ability of prostate cancer cells even at low doses (Gumushan Aktas & Akgun, 2018).

21.2.3.4 Flavanols, flavan-3-ols, or catechins

Epigallocatechin-3-gallate reduces the migration, invasion and metastasizing abilities of several cancer cells, such as melanoma cancer cells (Zhang, Lei, et al., 2016), bladder cancer cells (Luo, Lung, Chun, Luo, & Huang, 2018; Luo et al., 2017), human cholangiocarcinoma HuCC-T1 cells (Kwak et al., 2017), CAL27 and SCC 15 tongue squamous cell carcinoma cells (Li, Gu, et al., 2018), through suppression of MMP-2 and MMP-9 expression, downregulation of NF-κB expression, and inhibition of cell proliferation, inducing apoptosis with a dose- and time-dependent manner in both in vitro and in vivo. 25 μM EGCG inhibited migration and invasion through decreasing expression levels of MMP-9 and NF-κB in both SW780 human bladder cancer cell lines and BALB/c nude mice (Luo et al., 2017).

When (+)-catechin covalently conjugated with gelatin (gel-CT), a catechin nanohybrid, induced cell death inhibited cell proliferation, migration, and neoangiogenesis with a decreasing IC_{50} value of (+)-catechin in both bladder cancer cell lines and zebrafish embryos (di Leo et al., 2017).

21.2.3.5 Isoflavones

Genistein inhibits the migration, invasion, and metastasis abilities of cancer cells, including melanoma cells, colon cancer cells, and ovarian cancer cells. When genistein was applied to melanoma cells, the adhesion, migration, and invasion abilities of melanoma cells were suppressed through inhibition of FAK, paxillin, vimentin, and Snail mRNA levels and FAK/paxillin and MAPK pathways (Cui et al., 2017). Studies demonstrated that genistein suppressed MMPs and TGF-β signaling, resulting in inhibited EMT and migration ability of BG-1 ovarian cancer cells (Kim, Choi, & Hwang, 2015), and genistein reverses EMT by suppression of EMT-related markers such as N-cadherin, Snail2/slug, TWIST1, FOXC1, FOXC2, ZEB1, and ZEB2 in HT-29 colon cancer cells (Zhou, Wang, et al., 2017). When genistein was administered with doxorubicin-loaded nanoparticles, the side effects of free doxorubicin were reduced and genistein inhibited the metastasis at the same time through regulating ROS generation and ROS-induced oxidative damage in malignant prostate cancer models (Wang, Zhang, et al., 2018).

21.2.3.6 Anthocyanins

75 μM delphinidin inhibits invasion and migration through inhibition of EMT markers and ERK/p38 MAPK-signaling pathway in human osteosarcoma cell lines, as well as inducing mitochondrial-mediated apoptosis (Kang, Park, et al., 2018). However, delphinidin inhibits EGF-induced EMT and suppresses the invasion and migration through regulating Snail, MMPs and EGFR, AKT, and ERK signaling pathways with a dose-dependent manner in hepatocellular carcinoma cells (Lim, Kim, & Ko, 2018). Another study showed that delphinidin (50 and 75 μM) inhibits brain-derived neurotrophic factor (BDNF)-induced migration and invasion in ovarian cancer cells through inhibiting MMP-2 and MMP-9 expression (Lim, Kim, et al., 2017).

21.2.4 Flavonoids in reversal of drug resistance

Development of resistance against chemotherapeutics is a major problem in cancer treatment. The studies are focused on the reversal of drug resistance in cancer and new treatment applications are developed to overcome this problem. Flavonoids are one of the therapeutic tools that can be used to reverse resistance by regulating different resistance mechanisms. In this part, recent studies which showed the reversal of drug resistance by flavonoids families in several cancer types were summarized.

21.2.4.1 Flavones

Luteolin efficiently overcomes paclitaxel resistance by suppression of EMT which is upregulated in resistant ovarian cancer cells (Dia & Pangloli, 2017).

Apigenin decreases or inhibits drug resistance in cancer cells as well as enhancing efficiency of chemotherapeutic drugs. Studies demonstrated that 80 μM apigenin exposure to Adriamycin (ADR)-resistant MCF7 breast cancer cells overcome ADR resistance and increase intracellular drug accumulation through regulating P-gp (MDR1) multidrug resistance (MDR) mechanism and STAT3 signaling pathway (Seo, Ku, et al., 2017).

A citrus pentamethoxyflavone, tangeretin reverses the ABCB1-mediated multidrug resistance in human PTX-resistant A2780 ovarian cancer cells and A549 nonsmall cell lung cancer (NSCLC). Moreover, it has a long time reversal effect against ABCB1-mediated multidrug resistance when it is used together with PTX. Docking analysis

demonstrated that tangeretin interacts with the ABCB1 binding site and prevents its efflux activity (Feng et al., 2016). This study is promising for combination therapy of tangeretin and PTX in clinics.

21.2.4.2 Flavonols

Quercetin can reverse the resistance in cancer cells. A study on KB/VCR oral cancer cells which have drug resistance against vincristine showed that applying an increasing dose of quercetin inhibited the P-glycoprotein (P-gp)-mediated MDR (Yuan, Wang, et al., 2015). Other supporting studies showed that quercetin enhances the efficiency of gemcitabine on lung cancer cells through inhibition of heat shock protein 70 (HSP70) (Lee, Lee, et al., 2015) and enhances the effect of doxorubicin against breast cancer cells and eliminates breast cancer stem cells, while it was nontoxic on the nontumorigenic breast cell line (Li, Yuan, et al., 2018).

Another study demonstrated that 20 μM fisetin augments the chemotherapeutic effect of 5 nM cabazitaxel on prostate cancer cells through decreasing cell proliferation, colony formation, and promoting apoptosis in PCa cells; inhibiting tumor growth, metastasis, and its protein ex in athymic nude mice. In addition, fisetin decreases P-gp expression in P-gp expressed-NCI/ADR-RES cells and they suggest that the findings reflect that fisetin can be a novel microtubule-stabilizing agent that can be used in the reversal of drug resistance of other cancer types (Mukhtar, Adhami, Siddiqui, Verma, & Mukhtar, 2016).

100 μM kaempferol reverses the drug resistance and promotes apoptosis through inhibition of ABCB1 and ABCC1 genes expression that are involved in drug resistance in human HL60 and NB4, and polymorphonuclear cells (PMNs). They also demonstrated that kaempferol and all-*trans* retinoic acid (ATRA) induces differentiation of primary acute promyeloid leukemia (APL) cells (Moradzadeh et al., 2018).

21.2.4.3 Flavanones

Flavanone reduces and reverses drug resistance in prostate cancer and ovarian cancer cells. When paclitaxel is applied to cells with naringin, the cytotoxic, apoptotic, and antimigration effects of paclitaxel were increased through inhibition of Nf-kB and upregulation of PTEN by naringin in PC3, DU145, and LNCaP prostate cancer cells. The combination with docetaxel showed similar results in these cell lines (Erdogan, Doganlar, Doganlar, & Turkekul, 2018). Inhibition of expression of Nf-kB and P-gp by naringin led to the reversal of cisplatin resistance in ovarian cancer cells (Zhu, Gao, Wang, Qian, & Cai, 2018).

21.2.4.4 Flavanols, flavan-3-ols, or catechins

A green tea polyphenol, EGCG, reverses doxorubicin-resistance through decreasing P-gp and MDR1 overexpression, as well as blocking PI3K/Akt and MEK/ERK signaling in HepG2 hepatocellular carcinoma cells (Satonaka et al., 2017). Another study showed that EGCG can also reverse multidrug resistance against adriamycin (ADM) through significantly decreasing ABCG2 expression and inducing apoptosis in MDR-esophageal cancer cells (Eca109/ABCG2) (Liu, Ju, Wang, & Zhou, 2017). Not only does EGCG reverse drug resistance but also its derivate Y6 (5,3′,4′,3″,4″,5″-6-*O*-ethyl-EGCG), which is an ethylation yield of EGCG, reverses doxorubicin resistance of human hepatocellular carcinoma cells through decreasing Mdr1 and hypoxia-inducible factor-1α (hif-1α) expression (Wen et al., 2017).

21.2.4.5 Isoflavones

Genistein did not show reversal effects to drug resistance that occurs through regulation of ABCC2 (ATP Binding Cassette C2, multidrug resistance protein 2, MRP2) and ABCG2 (ATP Binding Cassette G2, breast cancer resistance protein, BCRP) in colon colorectal cancer cells (Schexnayder & Stratford, 2015). Also, genistein did not show an enhancer effect for taxane chemotherapy against the tumor growth of castration-resistant prostate cancer, although genistein inhibited P-gp-mediated drug efflux (Eskra, Schlicht, & Bosland, 2019). Unfortunately, another study showed that genistein enhanced multidrug resistance through translational regulation of ABCC1 and ABCG2—ABC transporters—and reducing chemotherapeutic drugs efflux in breast cancer cell lines (Rigalli et al., 2016).

21.2.5 Flavonoids in angiogenesis

Cancer cells can spread to other distant organs and build new blood vessels and a vascular network, known as angiogenesis. Angiogenesis is an important issue because cancer cells need oxygen and nutrients for supply. In this part we review recent studies which have revealed the effects of several flavonoids on the angiogenesis ability of cancer cells.

21.2.5.1 Flavones

Luteolin inhibited angiogenesis and vasculogenic mimicry formation by suppression of VEGF and Notch1 expression as well as inhibited migration and cell proliferation in gastric cancer (Zang, Hu, Zhang, et al., 2017), while luteolin suppressed progestin-induced VEGF mRNA expression and secretion, decreased expression of angiogenesis markers and stem cell-like properties in breast cancer cells, and also inhibited tumor growth in breast cancer xenograft mice (Cook et al., 2015).

21.2.5.2 Flavonols

Quercetin inhibits angiogenesis through upregulation of thrombospondin-1 expression and protein levels as well as inhibiting invasion and metastasis in PC-3 human prostate cancer cells and BALB/c nude mice (Yang, Jiang, et al., 2016).

Application of myricetin inhibits angiogenesis, which is an important event in cancer progression, through doenregulating the PI3K/Akt/mTOR signaling pathway in human umbilical vascular endothelial cells (HUVECs) (Kim, 2017). Also, the combination of myricetin with galangin inhibited angiogenesis through inhibition of vascular endothelial growth factor (VEGF) and reduced Akt phosphorylation and hypoxia-inducible factor-1α (HIF-1α) protein level in ovarian cancer (Huang, Chen, et al., 2015).

21.2.5.3 Flavanols, flavan-3-ols, or catechins

(−)-Epigallocatechin-3-gallate (Pro-EGCG) inhibited angiogenesis through suppressing VEGFA secretion by inhibiting PI3K/AKT/mTOR/HIF1—a signaling pathway in xenograft model of endometrial cancer (Wang, Man, Chan, Kwong, & Wang, 2018). In addition, (−)-epigallocatechin-3-gallate inhibited microenvironment-induced angiogenesis, migration, and invasion through preclusion of the JAK/STAT3/IL-8 signaling pathway in colorectal carcinoma by a combination of EGCG and curcumin (Jin et al., 2017).

21.2.5.4 Anthocyanins

Delphinidin inhibits angiogenesis through the inhibition of HIF-1α and VEGF expression in lung cancer (Kim et al., 2017), while delphinidin suppresses VEGFR that leads to inhibition of angiogenesis and tumor growth in endothelial cells (Keravis et al., 2015).

21.2.6 Flavonoids and the stem cell properties of cancer cells

Cancer stem cells or tumor initiating cells are found in cancer heterogeneity as a subpopulation and they corroborate cancer cells against chemotherapy or radiotherapy, regulate the metastasizing ability of cancer cells, and cause relapse during treatment. For these reasons, cancer stem cells should be targeted by current or future therapies. Flavonoids can be used to target cancer stem cells by inhibiting signaling pathways that are related to CSC properties of cancer cells. Targeting of cancer stem cells by flavonoids has been reviewed by Cianciosi et al. (2018).

21.2.6.1 Flavones

It was documented that luteolin suppressed oral cancer stem cells (OCSC) (ALDH + /CD44 +) through decreased self-renewal ability of OCSC, as well as enhanced radiosensitivity and inhibition of IL-6/Stat3 signaling (Tu et al., 2016), while luteolin inhibits stem cell−like properties of human breast cancer xenografts (Cook et al., 2015). Luteolin showed synergetic effects with silibinin against glioblastoma and glioblastoma stem cells with inhibited invasion, migration, and angiogenic factors (Chakrabarti & Ray, 2015). The same group also showed that this synergetic effect causes morphological changes, inhibiting invasion, metastasis, autophagy, and angiogenic formation as well as inducing apoptosis and overexpression of miR-7-1-3p in glioblastoma xenograft nude mice (Chakrabarti & Ray, 2016). Proteomics analysis revealed that luteolin suppressed stemness of prostate cancer cells through regulating Wnt, TNF, PI3K/AKT, MAPK, VEGF, HIF-1, Hippo, FoxO, and mTOR signaling pathways, especially Wnt suppression by FZD6 (frizzled class receptor 6) upregulation. Luteolin also suppresses cell markers including CD44, CD133, OCT4, and BMI1 of PC-3 spheres, and inhibits the proliferation and migration of PC-3 cells (Han, Lang et al., 2018).

Apigenin reduces the CD44 + /CD24 − subpopulations of triple-negative breast cancer cell lines through regulation of the YAP/TAZ signaling pathway which contributes to stem cell properties of cancer cells, such as stemness, resistance to chemotherapy, and metastasis (Li, Xu, et al., 2018). Another study showed that apigenin enhanced the cisplatin activity against prostate cancer stem cells when apigenin was combined with cisplatin (Erdogan, Turkekul, Serttas, & Erdogan, 2017). A recent study showed that hypoxia induced stem cell marker expression, while apigenin reduced the

cell viability and expressing-cell surface stem cell markers in human head and neck squamous cell carcinoma cell line (Ketkaew, Osathanon, Pavasant, & Sooampon, 2017).

21.2.6.2 Flavonols

Quercetin inhibits the gastric cancer stem cell growth and induces mitochondrial apoptotic pathways by the inhibition of PI3K/Akt signaling (Shen et al., 2016), while quercetin (dose- and time-dependent) inhibits proliferation, mammosphere, and colony formation of CD44 + /CD24 − cancer stem cells of MCF-7 breast cancer cells (BCSC). Also, quercetin inhibits tumor growth and metastasis ability of BCSC in nude mice model (Li, Zhou, et al., 2018). Another study showed that quercetin inhibits the survival and migration of prostate cancer stem cells (CD44 + /CD133 +), and induces apoptosis and cell cycle arrest by downregulating a growth factor midkine, PI3K/AKT and MAPK/ERK signaling pathways (Erdogan, Turkekul, et al., 2018).

Fisetin inhibits the proliferation and invasion ability of renal cancer stem cells from patient tissue samples and in mice through decreasing TET1 protein expression, which is involved in the 5-methylcytosine (5-mC) (a modified DNA base) to 5-hydroxymethylcytosine (5-hmC) conversion and belongs to the ten-eleven translocation (TET) protein family, as well as inhibiting TET1-induced CCNY/CDK16 promoter methylation (Si et al., 2018).

21.2.6.3 Flavanols, flavan-3-ols, or catechins

(−)-Epigallocatechin-3-gallate (EGCG) inhibits the cancer stem cells of lung cancer cells (Zhu et al., 2017), glioblastoma (GBM)(Farabegoli, Govoni, Spisni, & Papi, 2018), pancreatic ductal adenocarcinoma (Kumazoe et al., 2017) and colorectal cancer cells (Chen, Wang et al., 2017) through regulation of the Wnt/β-catenin signaling pathway which is involved in maintaining stem cell properties of cancer stem cells. When colorectal cancer spheroids were treated with different concentrations (10, 20, 40 μM) EGCG for 6 days, EGCG inhibited cancer stem cell properties and induced apoptosis through repression of Wnt/β-catenin signaling (Chen et al., 2017). In addition, lung cancer stem cells were suppressed by EGCG (Zhu et al., 2017). Another study revealed that EGCG inhibits the stem cell−like properties of lung cancer A549, H460, and H1299 cells and also patients serum samples through silencing hsa-mir-485-5p/RXRα that leads to the significant reduction of RXRα, CD133, CD44, Sox2, Nanog, E-cadherin, and MMP-9 expression. These results were also confirmed in A549 cell xenograft mouse model by EGCG (Jiang, Xu, et al., 2018). In addition, EGCG decreases cancer stem cells properties of pancreatic ductal adenocarcinoma cells both in vitro and in vivo when combined with phosphodiesterase 3 (PDE3) inhibitor (Kumazoe et al., 2017).

21.2.6.4 Isoflavones

Not only does genistein inhibit cancer stem cells-like properties, but also the genistein derivate 7-difluoromethoxyl-5,4′-di-n-octylygenistein reduces the stem cell−-like properties of ovarian carcinoma by the inhibition of AKT, ERK, and NF-κB pathways (Ning, Xu, Cao, Chen, & Luo, 2017), as well as inhibiting gastric cancer stem−like cells and reversing EMT gastric cancer (Cao et al., 2016).

21.3 Conclusion and future perspectives

Flavonoids are polyphenol secondary metabolites of plants which can be extracted from almost all parts of plants. There are different classifications of flavonoid families in the literature; however, flavonoids have their basic skeleton in common. Based on the differences in their substitution and activity of these carbon skeletons, flavonoids are divided into six classes: flavones, flavonols, flavanones, flavan-3-ols, isoflavones, and anthocyanins. Approximately 10,000 flavonoids are known up to now and several studies have supported the antioxidant, prooxidant, antiinflammatory, antiviral/bacterial, antidiabetic, cardio protective, anticancer, and antiaging effects of flavonoids families.

In this chapter, we reviewed the most common flavonoids families and recent in vitro and in vivo studies about their antiproliferative, antigrowth, apoptotic, antimigration, antiinvasion, antimetastatic effects, and also drug resistance reversal effects against several cancer types. Thousands of studies have documented that flavonoids have both anticancer effects against cancer cells, tissues or animal models through regulating several pathways which are regulated not only transcriptionally but also epigenetically in cells. According to the studies flavonoids have promising effects on cancer cells or tissues while not revealing any cytotoxic or side effects on healthy cells and/or tissues. In addition, in some studies about dietary intake of some flavonoids, their effects on cancer or healthy patients and nutrikinetics in the population have been documented, indicating that flavonoids can be used as chemotherapeutic or chemopreventive agents to reduce the risk of cancer (Jaskulski et al., 2017; Lee, Ng, et al., 2017; Reger, Zollinger, Liu, Jones, & Zhang,

2018; Scholl et al., 2018). It has been attempted to identify possible targets and mechanisms of flavonoids in cancer cells using bioinformatics tools in addition to cell culture experiments, tissue analysis, and organism-based studies (Luo, Guo, et al., 2018; Song, Zhang, Chen, & Lin, 2017; Xinqiang, Mu, Lei, & Mun, 2017; Zeng, Shen, Gu, & Wu, 2018; Zhang, Yang, Ni, Teng, & Ning, 2018). Scientists have aimed to examine the target molecules of flavonoids, the interaction points between target molecules and flavonoids, and mechanisms of flavonoids which have anticancer effects on cancer and cancer stem cells. Considering the time and money cost, and to overcome possible problems in in vitro models, in silico models have been developed. In addition, flavonoid-loaded nanoparticles, liposomes, or other nanocarriers can open new windows in the future of flavonoids. Drug delivery systems enhance the effects of flavonoids through increasing the half-life of flavonoids in organisms. Thus the targeted therapies can combine with natural products, and harmful effects of chemotherapeutic agents can be tolerated by using new therapeutic strategies. Recent and ongoing studies support the idea of "targeted therapy" with natural compound "flavonoids." During chemotherapy, patients face some side effects of chemotherapeutic drugs such as losing weight, loss of hair, pains due to ototoxicity. A study showed that epigallocatechin-3-gallate protects the patients from ototoxixity (Borse et al., 2017). This study can open a horizon to use flavonoids as additive drugs to reduce side effects of chemotherapeutic drugs.

That is why flavonoid studies can be promising to use in drug development and clinics as chemotherapeutic, preventive, and additive drugs to enhance the effects of current chemotherapeutic drugs against cancer cells and tumors as well as reducing side effects of current drugs to healthy cells and tissues when some problems that are observed in preclinical studies are overcome. For example, although there are several beneficial effects and a wide range distribution of flavonoids, information about their bioviability and pharmacokinetics is limited (Wang et al., 2018). The bioavailability, the half-life in the body, interaction with other drugs and molecules, possible side effects, metabolization, and elimination methods of flavonoids should be examined to develop their use for chemotherapeutic or preventive drugs. Due to flavonoids being natural dietary compounds, therapeutically effective doses of flavonoids that are administrated against cancer or other diseases should be under careful consideration as well. Recent and ongoing studies aimed to modify the flavonoids to make compounds which are biologically available, easily deliverable, have no or low side effects, and specifically target cancer or tumor.

In addition, as we expected, flavonoids can show different cytotoxic effects on cell lines of the same cancer type as well as tissues due to cancer heterogeneity. Although, cell lines that belong to the same cancer types have similarities, due to cell lines being derived from different parts of the tumor or derived from different patients, different mutations occur which result in tumor heterogeneity; cell lines show different results in experiments. For instance, a study showed that quercetin showed different cytotoxic effects on different cell lines of human colonic cancer cell lines, HT29 and HCT15 (Raja et al., 2017). That is why, one cell line type of a cancer type does not reflect the whole cancer type and cell lines studies do not necessarily align with tissue or animal studies. It was shown that studies should not focus specifically one pathway to understand the cytotoxic effects of flavonoids on cancer cells (Eanes & Patel, 2016). Signaling pathways are related and communicate with each other, thus ongoing studies should be carried. Thus when moving from cell-based studies to organism-based studies, issues can be overcome. Moreover, a study about in vivo imaging of inhibitory effects of apigenin on hepatocellular carcinoma has revolutionized the idea that flavonoids can be used in in vivo imaging to monitor, characterize and quantify their biological effects (Li, Chi, Shao, & Fang, 2017).

As a conclusion, although the dietary intake of some flavonoids and their effects on cancer or healthy patients or their use as nutrikinetics in the population have been documented, the commercially available flavonoids, such as silibinin, galangin, hesperitin, genistein, naringenin, daidzein, taxifolin and celecoxib, can be used in chemotherapy or preventive therapy, when the questions about flavonoids and their effects within the body are answered.

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Chapter 22

Personalized biomedicine in cancer: from traditional therapy to sustainable healthcare

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22.1 Introduction

We will start with whether and how biomedical approaches are present and can improve sustainable qualified life. Providing a sustainable treatment application in cancer is a critical point for treatment. As is known, cancer is a big problem worldwide, and thus many scientific researchers are looking for a solution to get a complete cure for cancer. Different perspectives and approaches are found to prevent cancer progression. The main purpose of these approaches is targeting molecular pathways that are also important for personalized therapy.

The scope of drug discovery in cancer is very wide because every new piece of information about cancer progression provides a new perspective for drug discovery and development. Nowadays, the main treatment approaches are focused on epigenomics and epigenetic therapy, immunotherapy, targeted therapy, and pharmacogenomics for cancer therapy. All these topics are improved day by day. In addition to that, using computational biology is also important both for the determination of drug dynamic and kinetics as well as for experimental studies.

22.2 Biomedicine approaches in cancer

Cancer has an important place in the healthcare system and is a socioeconomic problem for society. This disease is the second leading cause of death in the world. According to data from the World Health Organization, 9.6 million deaths worldwide were estimated as being due to cancer in 2018. The diagnosis and treatment of cancer are crucial to increase the life quality of cancer patients and decrease the death rate. Understanding the causes and mechanisms of cancer will lead to achieving a better molecular medicinal approach for the diagnosis and treatment of cancer. Different perspectives are found in the field of drug discovery and development to determine new approaches to target cancer for treatment.

Some of the cancer research groups focus on targeting the oncogenes/oncoproteins that are the main drivers of cancer to initiate and progress cancer signaling pathways for cellular homeostasis (Califano & Alvarez, 2016; McManus, Chababi, Arsenault, Dubois, & Saucier, 2018). The mutations in the oncogenes induce dysregulation of the oncoprotein that causes many problems in cell growth, proliferation, and inhibition of apoptosis (Martincorena & Campbell, 2015; Tsatsanis & Spandidos, 2004). In such cases, healthy cells go through apoptosis (programmed cell death) but the expression levels of oncogenes are dysregulated to control apoptosis in cancer cells (Ichim & Tait, 2016). Different examples can be found in different cancer types. HER-2/neu oncogenes are a type of tyrosine kinase receptor that is called human epidermal growth factor receptors. HER-2/neu gene or protein is overexpressed in metastatic breast cancer. According to the literature, abnormalities in HER-2/neu can provide a prediction of disease outcome, that is, sensitivity or resistance toward chemotherapy drugs. In addition, HER-2/neu is used as an approach for targeted therapy with trastuzumab and follistatin (Park, Nedrow, Josefsson, & Sgouros, 2017; Seachrist et al., 2017). On the other hand, translocation between bcr and abl genes causes activation of the protooncogene that induces tyrosine kinase activation in human leukemia. Imatinib, as an inhibitor of tyrosine kinase, is used as a targeted therapy in leukemia (Huang et al.,

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2018). The myc, including c-myc, n-myc, and l-myc genes, that are also protooncogenes, regulate many cellular processes such as cell growth, proliferation, apoptosis, senescence, and cell motility (Kumari, Folk, & Sakamuro, 2017). The level of myc can be modulated by different targeting strategies, one of which is inhibition of the PI3K/AKT/mTOR signaling pathway that blocks myc translation by using Rapamycin, MK2206, and BEZ235 (Ilic, Utermark, Widlund, & Roberts, 2011; Mi, Ye, Liu, & She, 2015). Additionally, inhibition of myc protein at posttranscriptional level can be achieved by using the inhibitors against USP7, PLK1, and AURKA proteins (Chen, Liu, & Qing, 2018). Furthermore, different methods are being developed with developing technology to prevent abnormal expression of genes or proteins in order to target therapy of different cancers, such as RNAi and CRISPR technologies (Fellmann, Gowen, Lin, Doudna, & Corn, 2016; Pai et al., 2005). RNAi (RNA interference) is a mechanism which can be used to silence a gene expression. Cancer-related genes can be silenced with this method to prevent tumorigenesis or induce sensitivity of cancer cells to chemotherapy or radiotherapy (Pai et al., 2005). Within that context, small interfering RNA (siRNA) is used for sequence-specific gene silencing. This mechanism has been shown to be successfully applied in the mouse models (McCaffrey et al., 2002). The other mechanism is CRISPR/Cas-9 (clustered regularly interspaced short palindromic repeats) that is a relatively new genome-editing technique. Excitingly, this mechanism of gene editing is being used to educate the immune system to fight cancer cells (Liu, Zhang, Liu, & Cheng, 2017). Taken together, all these approaches are genome-based therapies that eventually will contribute to personalized medicine. The challenge is to be able to manage cancer-specific functions. The other important and new tool in cancer treatment is the regulation of epigenetic mechanisms. Nucleosome positioning, DNA methylation, and chromatin modifying are included within epigenetic mechanisms that regulate gene expression (Flavahan, Gaskell, & Bernstein, 2017). These epigenetic mechanisms are reversible to recover abnormalities in gene expression (Esteller, 2008). With epigenetic therapy, cancer cells are reprogrammed to inhibit cancer initiation and progression by using DNA methyltransferase and histone deacetylase inhibitors (Eckschlager, Plch, Stiborova, & Hrabeta, 2017). On the other hand, natural and synthetic compounds are also used as anticancer agents and chemopreventive compounds, known as the backbone of drug design for cancer therapy and drug delivery. Tubulin-binding agent in cell migration and topoisomerase inhibitors in DNA replication are the main natural cancer therapeutics, such as vinca alkaloids and taxanes, camptothecins and epipodophyllotoxins in cell migration and DNA replication, respectively, (Nobili et al., 2009). Also, nanoparticles are synthetic compounds that are used to deliver therapeutic and diagnostic agents to increase therapeutic efficacy and safety. Proteins, miRNAs, and synthetic polymers are conjugated with nanoparticles for both targeting and delivery (McMillan, Batrakova, & Gendelman, 2011). In addition, tumor metabolism, tumor microenvironment, and the interaction between cancer cells are other important points in biomedicinal approaches. Additionally, the level of nutrient, metal, oxygen, and intracellular metabolism of these molecules affects cancer cell progression and has also effects on the regulation of immune cells. So, a new innovative approach emphasizes nutrition traffic to regulate the cancer microenvironment and also the differentiation and activation of immune cells. To exploit this mechanism for cancer therapy, membrane transporters that are mediators to influx and efflux nutrition or waste can be targeted (Gouirand, Guillaumond, & Vasseur, 2018). Transporters are expressed differently in different cancers. So, a drug designer can focus on activation or inhibition of a specific transporter for a specific target.

Understanding cancer initiation and progression mechanisms of cancer cells helps to improve the evolution of new biomedicine approaches. However, at this stage, two questions need to be answered: "what do we know so far?" and "what do we need to know to develop better and more effective biomedicine approaches?" All cancer-related information can contribute to the development of biomedical research, health science, and the pharmaceutical industry.

22.2.1 Perspectives in the diagnosis and tracking of cancer initiation

Increasing the survival rate and life quality of cancer patients depend on, first, the determination of symptoms and second a good treatment. Cancer treatment can achieve success by accurate tracking of cancer dynamics using different tests and procedures. They provide confirmation about the presence of cancer and determination of the correct tumor type, location, and stage. Late diagnosis can cause a troublesome prognosis of cancer that also causes unsatisfactory therapeutic progress.

Tests for cancer diagnosis can be listed in four groups: physical, laboratory, imaging, and biopsy. The optimal treatment for cancer patients is provided by accurate diagnosis using clinical and histopathological tests. Molecular diagnosis puts forward a precise and objective diagnosis to cancer classification but currently some gaps still need to be filled, such as the molecular markers that are not specified and classified for some cancers yet. Specific antigens are used for the determination of cancer. For example, the level of prostate-specific antigen (PSA) increases in human serum during prostate cancer. The mortality rate in prostate cancer is extensive in the male. The early stage of pancreatic cancer is

not recognized because of mild or no symptoms. Enzyme-linked immunosorbent assay (ELISA), time-resolved fluorescence assay, chemiluminescent assay, surface-enhanced Raman scattering assay, and fluorescence immunoassay are used for the detection of PSA levels but they are sensitive to a high level of PSA which corresponds to the late stage of cancer. In addition, these analyses are done by using advanced and expensive instruments. Because of these reasons, a new electrochemical approach is being developed for high sensitization, selectivity, and low cost. Miao P. et al. worked with electrochemical reports of silver nanoparticle (AgNPs) to detect PSA levels, a procedure that is based on exonuclease-aided target recycling amplification. Their results also suggests that it can be applied in future clinical testing (Miao, Jiang, Wang, Yin, & Tang, 2018).

Tissue biopsy is a common diagnostic test for a cancer patient to identify the malignancy of a tumor. Nowadays, liquid biopsy is preferred to examine whether there is mutated DNA, exosome, or circulating cells in blood or in other fluids such as urine, saliva, ascites fluids, and pleural effusions (Macías et al., 2018). With the advancements in cancer research in recent years, new biomarkers such as circular RNA F-circEA that are produced by the EML4-ALK fusion gene are determined in nonsmall cell lung cancer (Tan et al., 2018). In addition to that, propeptides from collagen type III (PRO-C3) and collagen type VI (PRO-C6), both of which are tumor microenvironment proteins, are found to have roles in colorectal cancer. Serum level of these proteins increase in cancer suggesting that these biomarkers can be used for diagnosis and targeted treatment (Kehlet et al., 2018). Furthermore, specific microRNAs, which are small noncoding RNA molecules of 18−25 nucleotides, are found in different types of cancers. These microRNAs, which are expressed differentially in cancer cells, can modify signaling pathways to regulate cancer progression. Chen et al. first determined 69 miRNAs in serum of colorectal cancer patients. These highly expressed miRNAs are used as biomarkers for more sensitive and specific diagnosis (Chen et al., 2008). In addition to that, widespread miRNA profiling can be used for different types of cancer.

Next-generation sequencing (NGS), also known as massively parallel sequencing, is the novel technology for the diagnosis and risk prediction of cancer and cancer classification. NGS takes advantage of high-throughput sequencing of the large genome without needing any previous information of the subjected genome. Herewith, NGS is preferred in clinical and nonclinical studies instead of Sanger sequencing. Genetic screening for DNA or RNA mutations in different types of cancer is an important analysis of specific cancer qualitatively and quantitatively that also increases the applicability of personalized medicine (Kamps et al., 2017; Manogaran et al., 2018). The size of the DNA sequence data is increasing with the increasing number of bioinformatic studies. Therefore new algorithms are being developed for scalable machine learning approaches to determine the differences of DNA mutations in different types or stages of cancer. This knowledge has a vital role in the effective and precise results for the accurate diagnosis of cancer and determination of the quality of cancer cure (Brooks, Keating, Bergquist, Landrum, & Rose, 2018; Manogaran et al., 2018).

Early diagnosis of cancer provides the best chance for treatment to prevent cancer progression and develop planning individualized cancer treatment which could lead to a decrease in the mortality rate of cancer.

22.2.2 Novel approaches to prevent cancer progression and treatment of cancer

Cancer occurs by dysregulation in the balance of cell growth and cell death. Either an increase of cell proliferation or a decrease in cell death can induce cancer formation. The purpose of cancer treatment is triggering apoptosis (programmed cell death) in cancer cells by using internal or external factors without damaging healthy cells. So, the information about the signal transduction pathway in cell proliferation and apoptosis is important to understand cancer initiation and progression. The basic treatment focuses on two approaches which are the induction of apoptosis and/or inducetion of cytotoxicity directly. However, cancer cells are capable of gaining resistance to chemotherapeutic drugs. Therefore different approaches using epigenetic mechanisms, immune cells mechanisms, and targeted therapy are being developed to obtain 100% cure without leading to the generation of resistance (Fig. 22.1).

22.2.2.1 Drug discovery

The discovery and studying of new drugs are a significant process for the identification of new medicine approaches for the treatment of patients with cancer. The idea of drug design starts from the laboratory and reaches to clinic for treatment. Throughout this process, there are two main steps for using a drug in the clinic for treatment of cancer patients. One of which is drug application in vitro and in vivo to understand the effect of drugs in cancer cells and the other one is approval processes by the US Food and Drug Administration (FDA). The studies of drug application in vitro and in vivo take shape with three steps respectively which are preclinical research, clinical research, and postclinical

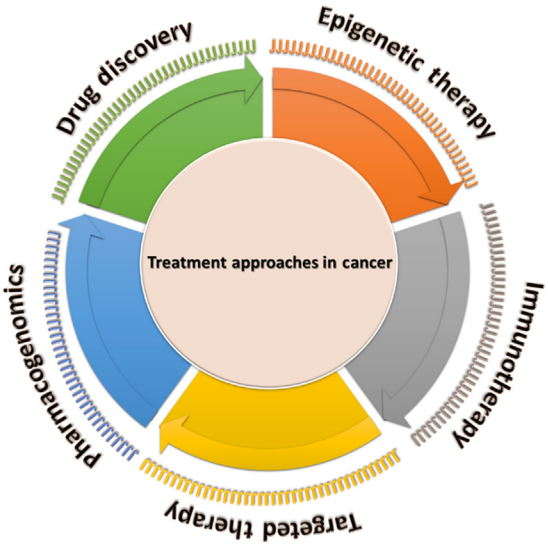

FIGURE 22.1 Novel approaches for the treatment of cancer.

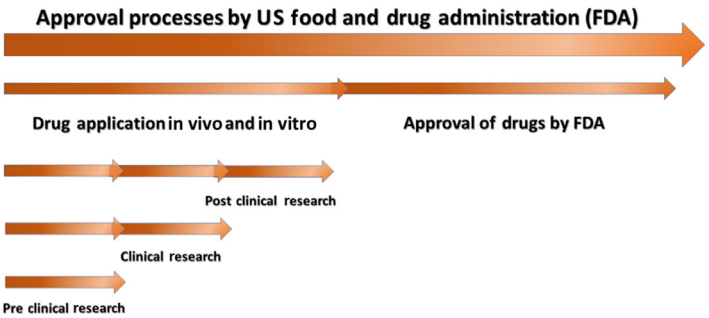

FIGURE 22.2 The processes of discovery and studying new drugs.

research. Each of these steps are the cornerstones of the drug development process (Fig. 22.2) (Faucette, Wagh, Trivedi, Venkatakrishnan, & Gupta, 2018; Wallach et al., 2018).

The discovery of a drug can happen in different ways. In the 1940s cancer chemotherapy started by using mustard gas during World War II (Smith, 2017). Mustard gas has an anticancer activity by DNA alkylation. After this discovery, other cancer drug applications have been developed by using the same concept of modification of DNA (Singh, Kumar, Prasad, & Bhardwaj, 2018). On the other hand, molecular mechanisms in specific cancer types are important to understand why different cancer types have different sensitivities and how to develop a new drug against the specific activity for cancer treatment. Many cancer-related genes such as bcr, abl, ras, raf, src, myc, etc. regulate signaling pathways in the cell cycle, apoptosis, angiogenesis, and metastasis. These cellular processes affect cancer progression (Fumarola, Petronini, & Alfieri, 2018; Tripathi, Liu, & Plattner, 2018; Zaravinos, Bonavida, Chatzaki, & Baritaki, 2018). The information about signaling pathways enlightens the field of drug discovery in cancer by the molecular mechanism-based approaches. Because the discovery and development of new, effective, and safe anticancer agents based on molecular-targeting.

Surgery, radiation therapy, and chemotherapy are common and effective methods for many cancer types. Traditional chemotherapy agents inhibit DNA replication and cell division to induce apoptosis. Many chemotherapeutic drugs such as dasatinib, imatinib mesylate, doxorubicin hydrochloride, taxanes, and capecitabine are used broadly for lymphocytic leukemia, breast, and colon cancer in clinical treatments (Cánovas et al., 2018; Fang, Fu, & Wei, 2018; Gleixner et al., 2018; Li et al., 2018). However, they have many serious side effects. Therefore complimentary treatment methods have gotten attention in clinical studies because of the background and disadvantages of chemotherapy. In addition to that, as mentioned before, cancer cells gain resistance that prevents sustainable drug application. Two important points which are how to predict drug resistance in cancer patients and how to prevent/overcome resistance, still await answers. Drug resistance mechanisms being examined to investigate new strategies are new medicinal chemistry technology of the use of molecular modeling to synthesize novel drugs and in pharmacogenetics by using a marker to determine resistance in patients.

In drug development in oncology, it is indicated that only 1 in 15 drugs will be approved by the US Food and Drug Administration to use in the clinical application. There are several reasons behind this low success rate (Hay, Thomas, Craighead, Economides, & Rosenthal, 2014). One is the transition from preclinical cancer models to the patient. Although there is success in the cell line models, the treatment might fail or have low efficiency in cancer patients. Potentially, cell lines and patient-derived xenograft (PDX) models are used to facilitate drug administration. Also, tumor organoid models are starting to be used to screen the effect of a drug in drug development in cancer. Tumor organoids are known as 3D cancer cell cultures that are derived from primary patient tumors and therefore provide better modeling under laboratory conditions (van de Wetering et al., 2015).

Cancer is a complex inherited disease but much information about the inheritance and genome of cancer has been discovered in recent years. CRISPR/Cas systems and RNA interference are known as recent developments that can edit and regulate a genome, providing a revolution in drug discovery and treatment in cancer because of the access to unlimited genetic mutation. Knock-out, inhibition, and activation of genes with CRISPR-Cas system are used to screen and identify their essential role in cancer that allows determination of the correct and better drug targets.

22.2.2.2 Epigenomics and epigenetic therapy

Gene and protein expressions are regulated initially by the DNA sequence. A mutation in DNA can be irreversible. In addition to the genetic information, the epigenetic code also controls the regulation of a gene and/or protein expression. However, the epigenetic code is heritable and reversible. Epigenetic mechanisms have important roles in the regulation of development, differentiation, and homeostasis of cellular processes. Modification of DNA by regulation of histone proteins and composition of nucleosomes is mediated through epigenetic mechanisms. Abnormalities in the epigenetic mechanism can cause genetic mutations that trigger cancer initiation and progression.

The regulation of gene expression and gene silencing in normal cells can be provided by DNA methylation that is mediated by histone modification (Curry et al., 2018). Histone modification also regulates chromatin structure (Reinberg & Vales, 2018). DNA methylation is one of the epigenetic modifications that is regulated by transfer of methyl group from *S*-adenyl methionine (SAM) to the C5 position of cytosine by DNA methyltransferases (DNMTs) to compose 5-methylcytosine. The addition of a methyl group in cytosine forms within CpG dinucleotides that are called CpG islands (Ye, Shi, & Li, 2018). CpG islands have a role in regulatory regions of the genome within many housekeeping gene promoter regions (Stirzaker et al., 2016; Takahashi et al., 2017). Aberrant DNA methylation is critical for cancer progression. In addition, it has been seen in many cancer types such as colon, breast, liver, bladder, Wilms, ovarian, esophageal, prostate, and bone (Jin & Liu, 2018). Therefore DNA methylation can be used as a diagnostic and prognostic tool (Hardy et al., 2017; Koestler et al., 2017; Xu et al., 2017). Moreover, suppressing the DNA methylation decreases tumor progression or induces sensitivity of cancer cells that are used in cancer treatment (Gursoy-Yuzugullu et al., 2017; Westerlund et al., 2017). Elliot Stieglitz et al. emphasized that DNA methylation can be used as a potential biomarker in the characterization of juvenile myelomonocytic leukemia (JMML) in addition to differences of age, fetal hemoglobin level, and the number of mutations in future clinical treatment and stem cell transplantation. With their findings, 75% of patients with a high level of DNA methylation were seen as relapsed posttransplant. So, it is suggested that the level of DNA methylation in JMML patients affects side effect and the success rate of stem cell transplantation (Stieglitz et al., 2017).

DNA methylation, histone modification and remodeling, and noncoding RNA regulate the organization of DNA into chromatin (De Majo & Calore, 2018). Long-range silencing of repetitive sequences, formation of silent heterochromatin, and transcription of DNA are maintained by these epigenetic mechanisms (Greenstein & Al-Sady, 2018). Epigenetic mechanisms in cancer can be directed by two main enzymes, which are DNA methyltransferase (DNMT) and histone deacetylases (HDACs). These processes are required for stability and conservation of cellular identity, cell proliferation and survival, apoptosis, and inflammation (Peng, Zhou, Zhang, Liu, & Zhang, 2018). DNA methylation is mediated by three main enzymes which are DNMT1, DNMT3a, and DNMT3b. These enzymes also maintain cancer development (Cai et al., 2017; Zhang, Lu, Wang, Yu, et al., 2018b). In addition, histone modification such as acetylation−deacetylation, phosphorylation−dephosphorylation, and methylation−demethylation regulates gene expression of DNA (Nowacka-Zawisza & Wisnik, 2017). Histone modification mediates DNA binding or transcriptional activity of the specific gene in cancer development and progression (Bradner, Hnisz, & Young, 2017). For epigenetic therapy in cancer, inhibitors of DNA methyltransferase (DNMT), histone deacetylases (HDACs), and anti-miRNA are used to target epigenetic mechanisms in different cancer types. These inhibitors can be used as a drug to target epigenetic regulators (Classon, LaMarco, & De Carvalho, 2017; Kurozumi et al., 2016; Siebenkas et al., 2017).

Minmin Liu et al. investigated the usage of DNA methyltransferase inhibitor guadecitabine (SGI-110) as a therapeutic for hepatocellular cancer. Guadecitabine is known as a second-generation DNA methyltransferase inhibitor that is also used in a clinical trial. The treatment of guadecitabine affects 48% of the gene in HCC that reduces tumor formation (Liu et al., 2018). In addition to that, Na Luo et al. determined the effect of guadecitabine on breast cancer. Guadecitabine enhances upregulation of major histocompatibility complexes (MHC-I) that induce antitumor immune response. Also, interferon stimulation and the expression of chemokines are mediated by guadecitabine in breast cancer patients. So, guadecitabine has been used as an epigenetic modifier in such studies (Luo et al., 2018). Azacitidine and decitabine are another two approved drugs for cancer therapy, as DNA demethylating agents. Also, Okochi-Takada put forward opinions about using DNA demethylating agents for high-throughput screening (Okochi-Takada et al., 2018).

Noncoding RNA (ncRNA) is a transcriptional product that is also called a regulatory molecule in many cellular processes. ncRNA plays an important role in chromatin remodeling, transcription, posttranscriptional modifications, and signaling pathways (Bo et al., 2015; Schmitt & Chang, 2016; Tang et al., 2017). Upregulation or downregulation of ncRNAs disrupts the signaling cascade in cancer cells. Cancer diagnosis and prognosis can be identified based on the expression levels of ncRNAs. In addition to that, ncRNAs are used as nucleic acid-based therapy tools in cancer (Arun, Diermeier, & Spector, 2018).

On the other hand, some compunds are used in epigenetic therapy such as polyphenol, flavonoid, organosulfur compounds, and cruciferous vegetables. They also modulate epigenetic mechanisms including DNA methylation, histone modifications, and expression of microRNA (miRNA) (Andreescu, Puiu, & Niculescu, 2018).

Any problem in the regulation of an epigenetic mechanisms can cause genetic alterations that dysregulate gene expressions that lead to the development of solid tumors and leukemia. The epigenome consists of chemical compounds that regulate gene expression by DNA methylation, histone modifications, and noncoding RNAs. Recent studies focus on the cancer epigenome to improve drug-actionable targets for specific treatments in cancer. Although the "Hallmarks" of cancer have not been contained in the role of the epigenetic mechanism in previous studies, recent studies emphasize epigenetic alteration induces cancer processes such as cell proliferation, invasion, migration, metastasis, and angiogenesis so this topic provides new perspectives for biomedicine.

22.2.2.3 Immunotherapy

The immune system is a defense system that consists of lymphoid organs, special cells, and substances to protect from infection and other diseases. Following the recognition of any foreign substance by the immunosystem, immune cells are attracted and remove the foreign substance by phagocytosis and keep regulating homeostasis in the body (de Visser, Eichten, & Coussens, 2006). Cancer cells escape immune cells because of the limitations of recognition as foreign in the body. These processes are maintained by receptors and immunosuppressive cytokines (He et al., 2007). Different immune therapy approaches are used because cancer cells are different from normal cells in terms of antigenic composition and biologic behavior. For this point, this system induced by monoclonal antibodies, immune checkpoint inhibitors, oncolytic virus therapy, T-cell therapy, nonspecific immunotherapies, and vaccines are the main types of immunotherapy. Different types of immunotherapy are used against different types of cancer and patients' immune system (Galluzzi et al., 2014; Rusch, Bayry, Werner, Shevchenko, & Bazhin, 2018).

In recent years, monoclonal antibody treatment has become one of the successful therapeutic strategies for solid tumors and hematologic malignancies. In the late 20th century, antibodies were called a "magic bullet" for cancer diagnosis and treatment (An, 2018). After the development of hybridoma technology, fluorescence-activated cell sorting (FACS), monoclonal antibodies (mAbs) are used to characterize cell surface protein of cancer cell. In recent years these approaches have been developed with different proteomic, genomic, and bioinformatic tools for the identification of cancer cells antigens. The selection of cancer antigen is important to determine both the biological roles of antigen in tumor growth and differences of the tumor and normal cells that play roles in antibody targeting and cancer therapy (Scott, Wolchok, & Old, 2012). Immunotherapy includes two main approaches which are antibody-based therapy, such as naked monoclonal antibodies, antibody−drug conjugates, and bispecific T cell engaging (BiTE) antibodies; and adoptive cellular therapies, such as chimeric antigen receptor (CAR) T cells. Monoclonal antibodies bind specific membrane-surface antigens to induce apoptosis with antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Phelan & Advani, 2018). Many special characterized and engineered humanized or chimeric MABs were approved by theFDA to target and kill the cancer cells. Alemtuzumab is used to target CD52 that is overexpressed in malignant lymphocytes, granulocytes, macrophages, and natural killer cells. Bevacizumab blocks the VEGF (vascular endothelial growth factor) receptor on cancer cells that provide inhibition of tumor blood vessels formation in the treatment of metastatic colon cancer and metastatic renal cancer, nonsmall cell lung cancer, and

glioblastoma (Hurwitz et al., 2004; McDermott et al., 2018; Michaelsen et al., 2018; Zheng et al., 2018). Cetuximab and panitumumab, which are used for metastatic colorectal cancer, head, and neck cancer patients, target and inhibit the activity of epidermal growth factor receptor (EGFR). They inhibit EGFR signaling pathways and induce cell death by ADCC and CDC (Trivedi et al., 2016; Wang et al., 2016). Ofatumumab and obinutuzumab are two novel anti-CD20 antibodies that destroy the distribution of lipid rafts on the cell membrane and increase the activity of CDC and ADCC which induces apoptosis at the end of the process. (Figueiredo, 2017; Pierpont, Limper, & Richards, 2018). Two monoclonal antibodies are used for targeting chronic lymphocytic leukemia. According to clinical studies, ofatumumab have beneficial effects on chronic lymphocytic leukemia patients. In addition to that, ofatumumab can be used with dexamethasone and cyclophosphamide as a combination therapy. (Wąsik-Szczepanek, Szymczyk, Kowal, Nogalski, & Hus, 2018). Trastuzumab is known as a humanized anti-ErbB2 monoclonal antibody that is used for breast carcinoma patients. Trastuzumab prevents HER2 dimerization on the membrane that causes the destruction of the HER2 receptor. This mechanism also induces ADCC. In addition to that, it stimulates anti-HER2 CD4 + T cells that recognize, bind, and kill cancer cells. Trastuzumab can be used with chemotherapy and reduces the rate of relapses (Clifton et al., 2017; Riccio et al., 2016). Antibody−drug conjugates consist of a combination of specificity of a monoclonal antibody (mAb) and a cytotoxic agent, targeted small molecule inhibitors, and targeted small molecule inhibitor for tumor-associated antigen target. Cytotoxic drugs such as doxorubicin, vinca-alkaloids, and taxoids kill cancer cells but they are limited in terms of selectivity and specificity which results in harming healthy cells too. In addition, naked monoclonal antibodies such as alemtuzumab, bevacizumab, cetuximab, ofatumumab, and trastuzumab are used with a cytotoxic agents to increase efficiency. Antibody−drug conjugates (ADCs) are developed to improve homogeneity and stability in pharmacological drug design (Beck, Goetsch, Dumontet, & Corvaïa, 2017; Ducry & Stump, 2010; Sievers & Senter, 2013; Trail, Dubowchik, & Lowinger, 2018). Bispecific T cell engaging (BiTE) antibodies contain a polyclonal T-cell response to target cell-surface tumor-associated antigens. The regulation of T-cell response in cancer cells is difficult to control tumor growth because cancer cells gain resistance to escape immune cells (Baeuerle & Reinhardt, 2009). CD8-positive T cells recognize cancer cells with the binding of major histocompatibility class I (MHC I) molecules on the cell surface to kill cancer cells. Blinatumomab (targeting CD19 and CD3) and catumaxomab (targeting EPCAM and CD3) were approved for use in acute lymphoblastic leukemia treatment and malignant ascites, respectively (Lameris et al., 2014; Yuraszeck, Kasichayanula, & Benjamin, 2017). Adoptive cellular therapies are tumor-infiltrating lymphocytes (TILs), specific T-cell receptor (TCR), and chimeric antigen receptor (CAR) T cells. The TILs are isolated from tumor tissue in patients and manipulated by stimulant to avoid tumor growth and provide regression.

Immunotherapy and personalized therapy have a relationship to develop precision medicine. Immune checkpoint inhibitors are used for therapy but the characterization of biomarkers in tissue or circulating system of patients is critical to control immune checkpoint mechanisms (Orabona, Mondanelli, Puccetti, & Grohmann, 2018). At this point, for the success in immunotherapy, identification of cancer and immune cells is of vital importance, as well as the development of biomarkers technology.

22.2.2.4 Targeted therapy

Targeted therapy is one method of precision medicine that induces the death of cancer cells without damaging healthy cells. So, targeting differs from chemotherapy in terms of the effecting mechanism and is the center of precision medicine and personalized therapy.

In targeted therapy, the main issue is identifying the targets. Firstly, the molecular differences between cancer cells and healthy cells are determined. Different protein families are found for targeting cancer cells, such as G protein-coupled receptors (GPCRs), ion channels, kinases, and nuclear hormone receptors (Bhullar et al., 2018; Bong & Monteith, 2018; Sherman, Downes, & Evans, 2012; Thomsen, Jensen, Hicks, & Bunnett, 2018). These proteins are involved in cell growth and proliferation. GPCRs are the regulators of cellular processes such as growth, metabolism, and homeostasis. Recent studies showed the involvement of GPCRs in tumorigenesis, cellular invasion, and metastasis (Liu et al., 2016). Targeting GPCRs is an efficient approach in cancer treatment. Especially, the therapeutic intervention is developed for targeting GPCRs in the sense of a relationship of ligand−receptor (Salon, Lodowski, & Palczewski, 2011). Ion channels mediate ion transport that regulates mitosis and cell migration by cell signaling pathways (Arcangeli & Becchetti, 2015). Particularly, Ca^{2+} channels or transporters provide homeostasis of Ca^{2+} in the cell. In the cancer cells, cellular processes such as gene transcription, proliferation, migration, and apoptosis can be dysregulated because of the problems in the transport of Ca^{2+} ions (Bose, Cieślar-Pobuda, & Wiechec, 2015). So, another approach has been developed with targeting Ca^{2+}-ATPase by an inhibitor (Tadini-Buoninsegni, Smeazzetto, Gualdani, & Moncelli, 2018). High levels of cytoplasmic Ca^{2+} ions induce the cell death pathways, such as apoptosis and necrosis

(Zhivotovsky & Orrenius, 2011). For example; the PMCA-selective inhibitor [Pt(O, O′-acac)(γ-acac)(DMS)] induces the increase of cytoplasmic Ca^{2+} ions that cause apoptosis in breast cancer by increasing the ROS effect (Muscella et al., 2011).

In cancer, the cell cycle process is also disrupted. In healthy cells, the cell cycle and cell cycle checkpoints are controlled by kinases which become constitutively active in cancer cells. Therefore getting the cell cycle under control by using the kinase inhibitors is an effective approach for cancer therapy. Cyclin-dependent kinase 4 (CDK4) and CDK6 are the main regulators of cell cycle in different stages (Lin, Zhu, & Ratner, 2018). Different CDK inhibitors such as flavopiridol, palbociclib, and abemaciclib are used as selectively targeted drugs (Kalra, Joshi, Munshi, & Kumar, 2017; Roskoski, 2018). In prostate and breast cancer, different drugs are used as inhibitors of androgen receptors and estrogen receptors, such as abiraterone and enzalutamide in the clinical treatment of prostate cancer and tamoxifen and letrozole for breast cancer (Bambury & Rathkopf, 2016). These drug applications are also called next-generation hormonal therapy (McDonnell, Wardell, & Norris, 2015; Watson, Arora, & Sawyers, 2015). On the other hand, DNA damage such as double-strand breaks (DSBs) or problems in homolog recombination can cause a serious problem in genomic integrity that also cause cancer formation (Gopalakrishnan et al., 2019). So, studies focus on proteins which mediate DNA repair system. The activity or inactivity of these proteins is rearranged by drugs for an appealing target of cancer cells. For example, MTH1 inhibitors TH588 and (S)-crizotinib are used as targeted anticancer drugs (Huber et al., 2014).

Furthermore, different drugs are identified as antitumor agents for cancer treatment but these drugs can be nonspecific and not as effective for cancer patients (Bahrami et al., 2017). This problem is solved by increasing the dosage and use-time of drugs to increase the effectivity of drugs on tumor cells (Alfarouk et al., 2015). However, two main problems are encountered after these methods of application. One is the prevention of drug delivery and the second one is direct transport of the drug to cancer cells. At this point, the nanoparticles are used as a carrier to target cancer cells specifically (Bahrami et al., 2017). Different types of nanoparticles are synthesized with suitable size and charge which are organic and inorganic (Cerqueira, Lasham, Shelling, & Al-Kassas, 2015). The use of nanoparticles creates a big impact on drug delivery with chemotherapeutic agents. The advantages of nanoparticles include decreased side effects, nonspecific uptake, undesirable target, and increased drug solubility and circulation time to improve drug efficiency and responsibility (Parveen, Misra, & Sahoo, 2012). However, nanocarriers have a limitation in the delivery of drugs to cancer cells. The extravasation of nanocarriers is one of the limiting factors (Miao & Huang, 2015). This problem is fixed by the characterization of physiochemical properties of the nanocarrier because the size, geometry, and charge of the nanocarrier affect the accumulation and extravasation of nanocarriers (Tan, Shah, Thomas, Ou-Yang, & Liu, 2013).

In conclusion, different drugs are used as inhibitors or blockers to induce cell death pathways. However, the cancer cells can gain resistance so any anticancer agents are not enough to overcome cancer. Therefore different approaches, such as specifically targeted drugs or modified nanocarriers, are developed for targeted therapy which is also important for personalized medicine and sustainable therapy for cancer patients.

22.2.2.5 Pharmacogenomics for cancer therapy

Genetic variation has a big role in any individual treatment in cancer treatment due to the variation in drug toxicity and efficiency. Pharmacogenetic studies show that drugs have different effects on different people, which are regulated by genes and environmental conditions (Madian, Wheeler, Jones, & Dolan, 2012). The transition of pharmacogenetics to pharmacogenomics studies brightened how to change pharmacokinetics and pharmacodynamics of cancer drugs in genetic variation of cancer treatment (Huang & Ratain, 2009). Also, pharmacogenomics researchers provide more understanding of cancer progression and response to drug application which is very important in personalized therapy (Schwab & Schaeffeler, 2012). Three main points that are important for the direction and development of pharmacogenomics can be listed as the determination of drug response in genetic variation, role of pharmacogenomics in drug discovery and development, and expectation in personalized therapy (Surendiran, Pradhan, & Adithan, 2008).

Many cancer types have multiple genetic abnormalities that make determination and characterization of the molecular profile even more important. According to genome profiling, patient-derived tumor cells are analyzed to assess drug sensitivity and resistance to predict clinical response (Zhang, Wang, Long, Liu, et al, 2018a). This approach creates a good infrastructure for drug screening and pharmacological profiling in cancer therapy. In addition to that, pharmacokinetics and pharmacodynamics are also key points to decide the optimal treatment approach and to decide the dosage and duration of use of anticancer agents for maximum efficacy and low toxicity (Barbolosi, Ciccolini, Lacarelle, Barlési, & André, 2015).

22.3 Development of drug discovery in biomedicine (model systems in drug discovery)

Many innovative approaches and perspectives are found for drug design and development that play important roles in transitioning biomedical studies into cancer treatment. Molecular information of cancer from whole cell analysis provides new molecular targets that eventually will lead to an improvement in cancer treatment. Predicted effective drugs have highly selective and low adverse effects. For this purpose, different innovative bioinformatic tools and software are used for drug design and discovery, adding new dynamic approaches to cancer treatment. The biggest challenge in drug discovery and development is the different properties of 2D and 3D models in vitro and in vivo.

22.3.1 Using computational models for drug design and discovery

Drug discovery and design processes require labor and a long time. Therefore many researchers in many pharmaceutical industries and medical research centers are directed to computer-assisted approaches (Drake, Knight, Harrison, & Søreide, 2018). Recently, bioinformatic tools of genomic, proteomic, and metabolomic approaches have brought more new dynamics for drug discovery and design than traditional approaches. So, computer-assisted drug discovery and design have an important role to develop and modify new therapeutic molecules or anticancer agents for validated targets and effective anticancer drugs (Nice & Baker, 2015; Wishart, 2016). Computer-assisted models provide a structure-based approach for drug design that ensures the prediction of structure, function, and experimental models of therapeutic molecules (Hassan Baig et al., 2016). Modeling and profiling of molecules such as determination and mapping of genome variation, interaction, and the relationship of proteins or noncoding RNA with the genome are shown by using computational models that serve the same purpose to determine the molecular information of cancer cells. In addition to that, overall any information in the field supports understanding of the disease mechanism (Cafarelli et al., 2017).

Thermodynamic and kinetic features are predicted by using molecular dynamics simulation. Free energy calculation and binding kinetics of drugs are used to make accurate and efficient predictions of drug function. Also, both ways provide an improvement of more effective and safe anticancer drugs (Tabeshpour et al., 2018; Zhao & Caflisch, 2015). In these days, machine-learning algorithms with developing computer science provision to determine drug binding mechanisms and kinetics are being widely used (Chiu & Xie, 2016; Decherchi, Berteotti, Bottegoni, Rocchia, & Cavalli, 2015). Moreover, allosteric site and mechanisms of drugs are determined by molecular dynamic simulation too. Identification of the allosteric site gives information about catalytic efficiency that is an important point for targeted therapy of cancer by increased drug−ligand selectivity and decreased drug resistance on cancer cells (Bhullar et al., 2018).

Pharmacophore is the concept of drug design to determine functional groups and binding sites of molecules such as hydrogen bond acceptors and donors, and aromatic and hydrophobic groups that provide information about the biological response and interaction of biological molecules (Haseeb & Hussain, 2015).

Different software and tools are used to the predict the properties such as absorption, distribution, metabolism, excretion, and toxicity of a drug (Jing et al., 2003). QikProp, StarDrop, and VolSurf + are commercially available softwares and PK/DB, admetSAR, and pkCSM are some of the online tools that can be used for different purposes in drug design process (Cheng et al., 2012; Cruciani, Pastor, & Guba, 2000; Moda, Torres, Carrara, & Andricopulo, 2008; Peach et al., 2012; Pires, Blundell, & Ascher, 2015).

The development process of genomics and high-throughput screening technology is very fast and can provide a large amount of data analysis. So, this tool leads to the emergence of a new trend which is "data mining." New research on the integration of computational methods and artificial intelligence approaches demonstrate how to get ahead of failures in a drug trial. In addition to that, the structure, function, and modulation of drugs are predicted by using the innovative approach of computational chemical biology and artificial intelligence (Mak & Pichika, 2018; Patel et al., 2009). Use of the computational methods plays an important role in increasing targeted therapy approaches by showing the relationship between drugs and diseases. At this point, the action between drug and target molecules becomes highly critical in drug designing process.

22.3.2 Pharmacological modeling and in vitro testing

Cancer is a highly complex and heterogenous disease and different in vitro models are used to understand the progression of cancer and development of approach in medicine. Cancer cell lines are used to mimic the tumor environment

for anticancer drug testing. In vitro and ex vivo models are critical steps for novel drug discovery studies. The problem in preclinical studies is that most of the time the efficiency of novel drugs in clinical application decreases during clinical trials (Pauli et al., 2017). To overcome this problem, new 2D and 3D cell line models are developed and improved to increase the effect and safety of drugs. The 2D model is an initial model developed to be used in drug application. In 2D models, cells are grown as a monolayer. This model has limitations such as not having cell−cell interaction and cell−extracellular matrix interaction. Therefore this limitation led to the formation of a new alternative model that is now used, called the 3D model. In the 3D models, cells grow in three dimensions. This model is more suitable for mimicking the tumor microenvironment. Cellular processes such as angiogenesis, metastasis, invasion, and migration are demonstrated with lifelike results by using 3D cell line models. Moreover, drug sensitivity and cytotoxicity can be detected differently in 3D models compared to 2D models. This difference happens because of the dispersion of drugs on cells. Also, the experimental results showed that targeted drugs or inhibitors such as anthracycline, docetaxel, and A83-01 (TGF-βR inhibitor) are more effective in 3D models (Fang & Eglen, 2017; Horvath et al., 2016).

New 3D models are established as bioreactors in the controlled cell culture system. This dynamic system adjusts the physiological variables such as oxygen, pH, and temperature that also affect drug response in the cell culture environment. Providing nutrition, cytokines, O_2, and growth factors, removing waste and reducing stress and pressure in the circulation system are important points for the sustainable and accurate drug application in in vitro models. There are three different dynamic systems which are stirred culture vessels, rotary cell culture systems, and microfluidic devices (Garreta et al., 2017; Gupta et al., 2016).

On the other hand, tumor tissue slice models are another in vitro model. The tumor microenvironment is protected from other types of cells. This model is one real way to show tumor biology that also affects drug sensitivity. Furthermore, some results demonstrate that the samples of tumor tissue slice culture are close to patients' solid tumor variabilities to compare immortalized cell lines because they are less differentiated (Hickman et al., 2014). Drug response with proliferation, progression of cell death, and drug uptake is investigated by gene and protein expression levels. The role of immune cells on tumor cells, such as migration, differentiation, and localization are examined by this model (ex vivo) that is also suitable for the investigation of immune and gene therapy approaches (Gajewski, Schreiber, & Fu, 2013). Tumor heterogeneity is one of the limitations to catch accurate equilibrium on inter- and intratumoral distribution. So, taking samples from different sections of a tumor is necessary for reliable results. In addition to this situation, optimized method should be found and established for different individual tumor tissue slide models.

In vitro model has an important role in understanding the effect of drug response for drug discovery and development that is mediated by signaling pathways. Additionally, biomaterials, microfluidics, and tissue engineering are developed to improve different 2D or 3D in vitro models that provide and obtain quality and applicable cell culture.

22.3.3 Using in vivo models for drug development

The use of in vivo models is a critical point to predict the effect of anticancer drugs that play important roles in drug development and future clinical treatment. In addition, the correlation between preclinical and clinical treatment is a remarkable topic for the prediction of tumor response as resistant or sensitive for future cancer treatment. The tumor has a heterogeneous structure and dynamic microenvironment that includes malignant cells, stromal structure, immune cells, cytokines, and growth factors (Gao et al., 2015). Drug discovery research is performed by using in vitro models that are based on cell lines but sometimes the results obtained using in vitro models cannot be represented in the actual tumor (Katt, Placone, Wong, Xu, & Searson, 2016). To mimic the complex and dynamic structure of the tumor in the patients better in laboratory conditions, coculture, ex vivo, and in vivo models are used to maintain the development of new strategies for drug applications (Bray et al., 2017; Dhami, Kappala, Thompson, & Szegezdi, 2016).

During the preclinical studies, cellular progression such as cell proliferation, growth, and apoptosis are shown by using 2D cell line models. However, cell−cell and cell−environment interactions are more effective for screening in vivo. In addition, organoids that are more complex in vitro models are patient-derived cells in the enriched medium within the extracellular matrix. The cells grow in three dimensions providing an advantage for drug response (Baker, Tiriac, Clevers, & Tuveson, 2016).

There are different approaches in a model organism to understand cancer biology, mimic human cancer biology on the model organism, and develop and achieve reliable cancer treatment. For this purpose, genetically engineered mice-derived models and patient-derived xenograft models are commonly used. In the patient-derived xenograft (PDX) model, tumor that is derived from a human is transplanted into an immunosuppressed mice, whereas transplanting cell lines that are originally derived from a patient into immunosuppressed mice is called cell line-derived xenograft (CDX) model. The effect of chemotherapeutic drugs and inhibitors on cancer cells are determined by using these models.

Additionally, genetically engineered embryonic stem cells are transplanted to fully immunocompetent mice so chemotherapeutics, drugs, and inhibitors, as well as immunotherapeutic agents, are detected by using genetically engineered mice (GEM) models (Choi et al., 2018; Xu, Li, Liu, Li, & Luo, 2019).

Many types of research show that mice and humans carry significant levels of similar features so mice models are used for the therapeutic development of preclinical studies. However, one might argue that using mice is not as suitable for use in therapeutic development. Instead, the GEM model is preferable to investigate immune systems and drug metabolism. Using PDX and GEM allow efficient, accurate, and reproducible data to be obtained.

22.4 The future direction of biomedicine for healthcare and conclusion

The information about cancer biology is increasing from the development of technology and many different approaches can be found for cancer diagnostics and treatment. In addition, cancer is a more complex disease in terms of structure, function, and mode of action in the human body. For example, heterogeneity, intra- and interstromal structure, tumor microenvironment, metabolism, and immune response all cause a challenge in preclinical and clinical achievement.

Understanding the biology of cancer brings new approaches and perspectives for a new generation of molecularly targeted drugs that are the cornerstones of personalized therapy. Four main therapies—epigenetic therapy, immunotherapy, targeted therapy, pharmacogenomics for chemotherapy—as well as chemoprevention have the potential to fill the gaps in biomedicine.

On the other hand, biomedicine research in the world is gaining a different perspective by the development of technology. "The Human Genome Project" is a milestone for sustainable knowledge. Also, this project suggests that not only experimental research but also bioinformatic studies are necessary to organize the massive data that help to manage data analysis and trajectories in biomedical sciences.

In conclusion, despite advances in technology and scientific findings in the industry, 100% cure is still not available for different cancer types. Different perspectives and approaches have been developed to provide efforts toward revealing better cancer treatment routes. So, we need to change our perspective and be open to new technologies to see the unseen.

> *"Thus the task is not so much to see what no one yet has seen, but to think what nobody yet has thought about that which everybody sees."*
>
> Schopenhauer

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Chapter 23

Tumor-specific genetic profiling and therapy in biomedicine

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23.1 Changes in cancer cells in a tumor mass

Cancer cells are able to divide continuously without any control, rendering immortal cells with many changes in their DNA sequences. These changes cause the dynamic instability in their genomes. Therefore they have an edge over other cells upon their alterations in marker expression, marker clusters, and cellular signaling pathways and mutations in the checkpoints for cellular division and cellular growth-related genes or proteins and new synthesis of tumor-specific transcripts such as mRNAs and microRNAs. Moreover, the temporary or permanently epigenetic changes in the genome can direct the carcinogenesis in the tumor mass. These changes also aid cancerous cells to evade tumor suppressor proteins and immune system cells (Hanahan & Weinberg, 2011). Therefore each cancer cell in a tumor mass actually has various changes for the specific tumor type. These differences of cancer cells in the tumor mass of a patient will be discussed in this chapter.

23.1.1 Altered marker expression or differentiation

The cancer stem cell hypothesis states that tumor mass comprises differentiated cells called tumor-initiating cells. These cells can be defined as cells that are able to grow tumor mass or comprise new tumor mass (Zhou et al., 2009). According to the clonal evolution model that indicates the progressive differentiation of cancer stem cells (CSCs), tumor mass consists of various long-lived and progressively differentiated cells (Zhou et al., 2009; Zhu & Fan, 2018). As we understand, almost all cancer or healthy cells share a similar range of surface markers. However, the differentiated cancer stem or stem-like cells highly express surface markers such as CD133 (Abbasian, Mousavi, Arab-Bafrani, & Sahebkar, 2018; Wang, He, et al., 2015; Zhu & Fan, 2018), CD44 (Abbasian et al., 2018; Li, Ma, Zhang, et al., 2017; Zhu & Fan, 2018), CD24 (Abbasian et al., 2018; Li, Ma, Zhang, et al., 2017; Zhu & Fan, 2018), CD13 (Abbasian et al., 2018; Wang, He, et al., 2015; Zhu & Fan, 2018), and other functional markers such as ALDH1A1 (Li, Ma, Zhang, et al., 2017; Zhu & Fan, 2018). Therefore these cells have a stronger capacity to start tumorigenesis. The liver CSCs highly expressing both CD133 and CD13 transmembrane glycoproteins are able to increase the tumorigenesis rate in the healthy tissue compared to either CD133 or CD13-bearing CSCs (Wang, He, et al., 2015; Zhu & Fan, 2018). Another article states that tumor-initiating cells or cancer stem-like cells in colorectal carcinoma bearing either CD44 or CD133 surface markers give rise to a nearly twofold increase in colorectal tumor volume comparing to the $CD44^-$ or $CD133^-$ cells (Abbasian et al., 2018; Zhu & Fan, 2018). Indeed, the presence of both CD44 and CD133 in CSLCs increases nearly sevenfold the tumorigenic potential in colorectal carcinoma (Abbasian et al., 2018). Moreover, the presence of the CD133 biomarker promotes the expression level of CD44 in liver cancer cells and increases survival rate of circulating hepatic stem cells (Kim et al., 2018). Furthermore, expression level comparisons of CD24, CD44, and ALDH1 in four breast cancer subtypes indicate that the $CD44^+/CD24^-/^{low}$ ratio is related to malignancy level in these cancer subtypes. The highest CD44/CD24 ratio was detected in the basal mesenchymal (triple negative) subtype and this highest ratio shows powerful tumorigenic and proliferative capacities of this cancer subtype. When ALDH1 levels are compared in various long-lived and progressively differentiated cells, ALDH1 expression level is the highest in cells that have a stronger metastatic capacity. When CD44 and ALDH1 are knockdown separately or combined with

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siRNA in breast cancer, there is a significant reduction in tumorigenicity of breast cells in BALB/c nude mice (Li, Ma, Zhang, et al., 2017). Thus these cells can propagate and initiate tumorigenesis in tumor mass.

Besides the presence or absence of glycoproteins is important for tumorigenesis and/or metastasis, glycosylation modifications of proteins in cellular membrane cause the diversity within the tissue-generating glycosidic linkages between saccharides and protein (Pinho & Reis, 2015). Changes in glycan expression are a result of malfunction localization and expression of glycosyltransferases promoting the replacement of saccharides through acceptors, especially glycoproteins (Gill, Chia, Senewiratne, & Bard, 2010). It is indicated that the glycosylation process could play crucial roles in the tumor progression. The glycosylation process can be examined under five different branches: sialylation, fucosylation, *O*-glycan truncation, and N-glycan branching (Pinho & Reis, 2015).

Sialylated glycoproteins participate in cellular recognition, cellular signaling, and cellular adhesion (Li & Ding, 2018; Pinho & Reis, 2015). These sialylated glycoproteins have been exhibited to be apparently expressed and promote the tumor growth in various malignant cancers such as liver, ovarian, thyroid, prostate, and bladder (Li & Ding, 2018). The blockage of sialic acid suppresses the tumor growth stimulating the T cell-associated antitumor immunity (Bull et al., 2018). After sialidase treatment of lung cancer cells, results revealed that sialylation suppresses epidermal growth factor (EGFR) dimerization and the ligand-induced EGFR downstream pathway. An increase in fucosylated glycans synthesized by fucosyltransferases is a crucial marker modification in lung cancers. Lung cancer studies have demonstrated that overexpression in fucosyltransferase, as well as sialyltransferase, could suppress the EGFR dimerization and phosphorylation in EGF-treated lung cancer cells. Nevertheless, core fucosylation increase in EGFR by FUT8 causes the tumor growth promoting the EGFR dimerization (Liu et al., 2011). Besides, overexpression in FUT7 induces the fucose-required sialylated Lewis antigen (sLe^x), thereby promoting A549 cells via EGFR/AKT/mTOR pathway (Liang, Gao, & Cai, 2017). Similar to FUT7, FUT4 expression is increased in breast cancer cells and this increase can be suppressed by miR-200b by decreasing the fucosylation on EGFR and inactivating the PI3K/Akt signaling pathway. This suppression inhibits the tumor growth and metastasis ability of MDA-MB-231 and MCF7 cells and in vivo xenograft model (Zheng et al., 2017). Additionally, truncated *O*-glycans such as Tn and sialylated Tn form (sTn) and antigens T are increased in the breast, ovarian, and colorectal cancer. Lastly, tetraantennary N-linked glycans formed by N-acetylglucosaminyltransferase V (GnT-V) have been demonstrated in breast, hepatocellular, and colon cancers, and melanoma carcinoma (Christiansen et al., 2014).

Each tumor cell in a tumor mass has various differences in its markers, which affect the fates of cancer cells. The presence or absence of markers enhances malignancy of the cells by increasing their proliferation or metastasis abilities.

23.1.2 Altered intracellular signaling

Normal cells can be transformed into cancerous cells sourced by differences in the cellular signaling pathways. A tumor mass arises from various cancerous cells with changes in both glucose and lipid metabolisms to generate more energy. The Warburg effect claims that altered glucose metabolism in cancer cells promotes tumorigenesis by uptaking more glycose molecules, thereby affecting the cellular signaling through the modulation of both reactive oxygen species (ROS) and chromatin state. In ROS modulation, NADH is crucial for electron transport in mitochondria for ROS homeostasis. An imbalance in NADH concentration is reduced using NADH in de novo lipid synthesis to keep glycolysis active for tumor growth and proliferation rapidly. This highly active glycolysis process directly influences histone acetylation to stimulate the growth phase of cancerous cells (Liberti & Locasale, 2016).

Lipidomics studies revealed that cancer cells with higher proliferative capacity have increased lipid uptake, lipogenesis level, and cholesterol synthesis (Beloribi-Djefaflia, Vasseur, & Guillaumond, 2016). Additionally, lipid variety in cancer cells has influenced both lipid-related and tumor-specific genes. Overexpression of these genes such as stearoyl-CoA desaturase (SCD), ABCA1, AGPAT1, and ACSL1 can be stronger biomarkers for treatment of colorectal cancer patients (Vargas et al., 2015). The various lipid molecules in membranes of cancerous cells might be localized together to construct microdomains called lipid rafts. These drafts contain numerous signaling receptors and proteins predominantly participating in survival, apoptotic, prooncogenic, and metastatic pathways during the progression of tumorigenesis (Beloribi-Djefaflia et al., 2016; Mollinedo & Gajate, 2015). Moreover, tumor and stromal cells such as adipocytes, adjacent to tumor cells can interact and adipocytes can release free fatty acids through cancer cells for tumor growth. In this interaction, prostaglandins and sphingosine derivatives such as sphingosine-1 phosphate play crucial roles in tumor growth and tumor metastasis (Wang & Dubois, 2010). Besides, mouse model studies in colon cancer cells revealed that prostaglandin E2 stimulates carcinogenesis in colorectal tissue, cancer stem cell expansion, and metastasis through liver

tissue via NF-κB (Kim et al., 2017; Wang, Fu, Sun, Guo, & DuBois, 2015). Overall, these findings suggest that lipid metabolism alterations in cancer cells regulate the progression of tumorigenesis in many cancer types.

In addition to alterations in glucose and lipid metabolism, cancer stem cells have some changes in the signaling pathways. The first of these signaling pathways is the JAK/STAT signaling pathway, which is activated by the hormones, growth factors, and interleukins. This pathway regulates various cellular events in stem-like cells especially hematopoiesis (Stine & Matunis, 2013). The aberrant JAK/STAT signaling has been demonstrated in the stem cells of acute myeloid leukemia patients (Cook et al., 2014). In the glioblastoma research, inhibition of STAT3 in cancer stem cells decreased the proliferation and reduced the stem cell-related genes *Olig2* and *nestin* (Sherry, Reeves, Wu, & Cochran, 2009). Hence, it was revealed that STAT3 is highly active in breast cancer stem cells and its inhibition by using chemical agents reduced the proliferation and colonization of breast cancer stem cells (Zhou et al., 2007). Additionally, hematological and neurological expressed 1 like (HN1L) transcription factor activates STAT3 expression in breast cancer stem cells in triple negative breast cancer (Liu, Choi, et al., 2018). Furthermore, colon cancer stem cells and their adjacent cells in a tumor sample have highly expressed STAT3 protein and these stromal cells have secreted interleukin-11 significantly to activate JAK/STAT signaling. In colon cancer mouse model, highly active JAK/STAT signaling with TGF-β in colon cancer cells leads to their metastasis to liver cells. Therefore stromal cells can trigger colon cancer stem cells to metastasize through liver cells via the JAK/STAT signaling pathway (Calon et al., 2012).

Secondly, Hedgehog signaling is crucial for developmental progression of embryogenesis modulating the proliferation, migration, and differentiation. The roles of Hedgehog signaling in cancer stem cells have been revealed in numerous cancer types such as chronic myeloid leukemia (CML) and colon cancer (Merchant & Matsui, 2010). In a study of CML, the deletion of smoothened transmembrane receptor protein (SMO) dramatically reduced the CML stem cells number in *Bcr-Abl1*-induced CML. Besides, inhibition of SMO with chemical agents causes the reduced level of CML stem cell propagation. Conversely, SMO overexpression has increased by four times CML stem cells frequency and progression of CML in SMO-deficient mouse model (Zhao et al., 2009). Similar results were obtained in the downregulation of SMO in colon cancer stem cells and this article found out that the increased level of Hedgehog signaling activity stimulates the advance of the colon cancer. Hence, colon cancer stem cells can metastasize through liver cells with higher activity of Hedgehog signaling (Varnat et al., 2009). When the Hedgehog inhibitors are applied to colon cancer cells in both in vitro and in vivo studies, they found that these inhibitors block SMO activity in both normal and resistant colon cancer cells to SMO inhibitors (Vesci et al., 2018).

Thirdly, Wnt signaling pathways with canonical and noncanonical manner are crucial for the tissue homeostasis. The canonical Wnt signaling modulates the proliferation and fate decisions while noncanonical signaling modulates cell polarity, asymmetrical cell division, and migration (Kahn, 2014). Mutations in genes coding proteins of the Wnt signaling have been documented in breast, colorectal, and gastric cancer and leukemia (Matsui, 2016). Numerous activators of Wnt signaling and downstream targets are upregulated or amplified in breast cancers but inhibitors of this signaling pathway are epigenetically silenced or inactivated (Incassati, Chandramouli, Eelkema, & Cowin, 2010). In a study of mammary glands of the mouse model, Wnt1 overexpression causes tumorigenesis and the number of cancer stem cells is more than six times higher compared to stem cells in the control group of mammary glands (Shackleton et al., 2006). On the other hand, mutations in or loss of the *APC* gene, a tumor suppressor, cause tumorigenesis in the intestine (Barker et al., 2009). Research into the interaction between surface markers and the Wnt pathway reveals that this pathway is highly active in $CD44^{+}/CD133^{+}$ colorectal cancer stem cells (Zhang, Huang, Li, Fu, & Xiao, 2016). Studies suggest that augmented Wnt signaling member activities or suppression of inhibitors or suppressors are highly crucial for breast cancer progression.

Fourthly, the Notch signaling pathway contains ligands and receptors, which are transmembrane proteins. Their interactions cause the cleavage of cytoplasmic domains of receptors to modulate stem or progenitor cell differentiation in hematopoietic and central nervous systems (Karamboulas & Ailles, 2013). In the literature, the Notch pathway is functional in various cancer types such as leukemia, breast, colon, pancreatic, and lung cancers. Pancreatic cancer stem cells isolated from patients have contained highly expressed Notch signaling genes such as *Notch1* and *Notch3* and *Hes1*, Notch target gene (Abel et al., 2014). When the inhibitors of Notch signaling pathway are applied to the cancer stem cell population of pancreatic tumor, the number of CSCs and tumor-sphere formation are decreased. These studies suggest that the Notch pathway is crucial for cell survival of pancreatic cancer stem cells (Abel et al., 2014). In addition to these signaling pathways in cancer and/or stem cells, PI3K, PTEN, and NF-κB pathways and other pathways are highly active or suppressed during tumorigenesis and advanced periods (Matsui, 2016).

Altered intracellular signaling causes the tumor progression from the first side of the tumor through the final step of tumor progression. The understanding of these pathways with the tumor-specific manner can aid in the development of individual tumor therapies in current biomedicine.

23.1.3 Synthesizing tumor-specific transcripts

Cancer transcriptome analyses revealed the numerous diverse transcripts unique to the cancer type. These transcripts are not only protein-coding RNAs but also noncoding members of the human genome, such as long noncoding RNAs and small noncoding RNAs, especially microRNAs or miRNAs. Both noncoding RNAs have crucial roles in cellular events such as proliferation, survival, and apoptosis. The long noncoding RNAs have more than 200 nucleotides whereas small noncoding RNAs have approximately 22 nucleotides.

Long noncoding RNAs are expressed in various cancer types and they are recruited in tumorigenesis and metastasis. Their aberrant expressions can contribute to using them as a potential biomarker in highly specific cancer types. As an example, lncRNAs such as PCGEM1 and PCAT-1 are overexpressed in prostate cancer to regulate cellular proliferation, chemoresistance, and metastasis respectively. Another mostly studied lncRNA, HOTAIR is overexpressed in various cancer types such as breast, gastric, and ovarian cancer. This lncRNA can regulate the progression of these cancer types from proliferation through the metastasis (Bhan & Mandal, 2016; Chang, Guo, Yuan, Shi, & Zhang, 2018; Feng & Huang, 2017).

MicroRNAs or miRNAs are small noncoding RNAs that regulate the gene expression. Their expression levels can be changeable in various cancer types upon the deletion or amplification of miRNAs, alterations in miRNA biogenesis, and epigenetic regulation. Thus miRNAs can participate in tumorigenesis or tumor suppression under tumor-specific conditions. In the tumor progression, miRNAs can be recruited in continuous proliferative signaling, survival, angiogenesis, and metastasis processes (Peng & Croce, 2016). MicroRNA-31 induces KRAS-linked lung tumorigenesis in lungs of mice models modulating negative regulators of the RAS/MAPK signaling (Edmonds et al., 2016). Additionally, miRNA-182 inhibits pyruvate dehydrogenase kinase 4 activity and stimulates lung tumorigenesis reprogramming de novo lipogenesis affecting JNK signaling (Li, Li, et al., 2017). On the other hand, overexpression of miRNA-145 inhibits tumor growth and invasions of cervical cancer cells by decreasing expression levels of core stem cell transcription factor in null-mice (Zhou, Yue, Wang, Gong, & Duan, 2017). Furthermore, miRNA-140-5p blocks the invasion and angiogenesis in breast cancer by negatively modulating VEGF-A in vitro and in vivo studies (Lu et al., 2017).

The tumor-specific transcripts can regulate the tumorigenesis, proliferation, invasion, angiogenesis, and metastasis negatively or positively. Their expression levels and interactions with other mediators in cellular metabolism are unique to the cancer types. Therefore their absence or presence is important for tumor-specific therapy in current biomedicine.

23.1.4 Evading from Immune system cells

Cancer cells are able to evade ımmune system cells by changing the reaction approaches, such as antiinflammatory cytokine production, T regulatory (T_{Reg}) cell induction, and expression levels of immune checkpoints such as PD-1 and CTLA-4 (Dyck & Mills, 2017).

Cancer cells can evade immune cells by regulating the immunosuppressive microenvironment releasing the proinflammatory cytokines. These cytokines aid the recruitment of myeloid-derived suppressor cells (MDSCs) and T regulatory cells. These cells produce the inhibitory cytokines such as Interleukin-10 (IL-10), IL-35 and transforming growth factor-β (TGF-β) to provide the mechanism of immune resistance (Pardoll, 2012). MDSCs are able to suppress the activities of NK cells and macrophages by blocking and polarizing, as well as inhibiting the T cell activation (Ostrand-Rosenberg, 2016). The last approach is the expression of the immune checkpoints. CTLA-4 is a CD28 homolog receptor protein that is highly expressed on the T cells and it mainly regulates the T cell activation. In this activation, T cell receptor (TCR) binds first the major histocompatibility complex (MHC), then B7-1 (CD80) or B7-2 (CD86) molecules on the antigen presenting cell (APC) binds with CTLA-4 receptor on the T cell, thereby leading to inhibitory signaling through T cell activation. There are two candidate CTLA-4 inhibitors, ipilimumab and tremelimumab to treat various cancer types such as lung, prostate, cervical, and colorectal cancers. However, these agents except ipilimumab in melanoma are not still approved in these cancer types and their status are still phase II or III (Buchbinder & Desai, 2016). The second immune checkpoint is PD-1 which is expressed on T cells to regulate T cell activation by binding to its specific ligands on tumor cells; programmed death ligand 1 (PD-L1) or programmed death ligand 2 (PD-L2). The binding of PD-1 to PD-L1/2 inhibits T-cell activation and this binding decreases T cell proliferation with production of interferon- γ (IFN- γ), IL-2 production, and tumor necrosis factor-α (TNF-α) (Buchbinder & Desai, 2016; Keir, Butte, Freeman, & Sharpe, 2008). There are three PD-1 associated PD-1-PD-L1 interaction inhibitors: pembrolizumab, pidilizumab, and nivolumab. Among these inhibitors, nivolumab is approved in the United States to treat the metastatic non-small cell lung cancer (NSCLC) but nivolumab and other inhibitors are still being studied in other cancer types. Similar to a PD-1 receptor, there are two PD-L1-associated PD-1-PD-L1 interaction inhibitors: durvalumab and atezolimab

whose status are phase II and III for some cancer types (Buchbinder & Desai, 2016). Additionally, it is newly documented about PD-L1 expression regulation that an RNA binding protein, LIN28, is upregulated in countless cancer cells and downregulates the biogenesis of miRNA let-7. This microRNA can posttranscriptionally inhibit the PD-L1 expression. This study has demonstrated that numerous cancer cells upregulate LIN28 expression to evade immune system cells via PD-1-PD-L1 interaction. The inhibition of LIN28 using an agent increased the let-7 expression and decreased PD-L1 expression, thereby resulting in the inhibition of tumor growth in vivo for promising treatments of various cancer types (Chen, Xie, et al., 2019).

Cancer cells are able to evade the immune system cells by negatively regulating them. Thus cancer cells can exploit the immunosuppressive state to grow and develop without any control. The approved inhibitors and other CTLA-4, PD-1, and PD-L1 inhibitors have huge potentials to construct future treatment options in the new age of biomedicine.

23.1.5 Evading tumor suppressor genes or proteins

The majority of genetic alterations in human cancers can be examined under two main categories: gain of function mutations in oncogenes that promote the cellular growth, survival, invasion, and metastasis and loss of function mutations in tumor suppressor genes that inhibit uncontrolled division and stimulate DNA repair mechanisms and activation of cell cycle checkpoints (Lee & Muller, 2010). There have been several tumor suppressor genes identified in various cancer types. First, retinoblastoma (RB) is the first identified tumor suppressor gene and mutated at an early stage in approximately a third of human cancers. Protein product called pRB is the limiting factor in cellular proliferation by negatively modulating E2F transcription factors, as well as regulating cellular fate, apoptosis, and chromosomal integrity (Dyson, 2016). Additionally, pRB inactivation downregulates epithelial cell motility and causes the loss of cellular polarity. However, these cancer cells can be motile upon loss of p53 activity or mutations in the p53 gene. Highly metastatic cancer cells such as retinoblastoma, SCLC, and osteosarcoma have demonstrated the commutation in p53 and pRB-1 (Parisi, Balsamo, Gertler, & Lees, 2018).

As a tumor suppressor protein, p53 is expressed at a low level in normal cells and is mutated in more than 50% of human cancers (Muller & Vousden, 2013). The cellular stress promotes the p53 posttranscriptional modification to activate p53, thereby binding to a specific DNA sequence to stimulate the activation of cell cycle checkpoints and senescence and apoptotic mechanisms. In the activation of cell cycle checkpoints, p53 targets CK1 p21 for G1/S and Polo-like kinase 1 for G2-M activation (Cicalese et al., 2009). While the p53 loss in somatic cells allows the reprogramming, p53 loss in mammary gland causes the asymmetric cell divisions of stem cells in this tissue and inactivation of p53 in cancer stem cells of mouse mammary tumors causes the symmetric cellular division (Cicalese et al., 2009; Lee & Muller, 2010). On the other hand, the tumor causing mutations in the *TP53* gene can be the missense mutations in the DNA binding domain of TP53 protein (Cerami et al., 2012). The lysine to arginine mutations (K117R + K161R + K162R) in p53 diminish its activity in cell cycle arrest, senescence, and apoptosis. These mutated p53 proteins in mice are able to activate the p53-related genes in the metabolic regulation (Li et al., 2012). Moreover, the reactivation of the p53 tumor suppressor protein significantly represses tumor progression (Wang, Suh, et al., 2011). Overall, p53 that is able to regulate the metabolism is a tumor suppressor protein (Liu, Zhang, Hu, & Feng, 2018).

Tumor suppressor proteins BRCA1 and BRCA2 are highly significant factors that regulate the DNA damage repair systems especially homologous recombination (HR) and checkpoints of the cell cycle. The main part of the BRCA1 sequence comprised by exon 11 interacts with BRCA2 via C-terminus of BRCA1 and PALB2 to construct the supercomplex for the tumor suppression (Jiang & Greenberg, 2015). Thus this complex stimulates the homology-directed repair via the formation of RAD51 nucleofilament and protects the replication fork, thereby maintaining genome stability. BRCA1 is also recruited by abraxas receptor-associated protein-80, RAP80, into the DNA damage site and activates signaling of the G2/M checkpoint (Kim, Chen, & Yu, 2007; Takaoka & Miki, 2018). BRCA1 and BRCA2 mutations are mainly demonstrated in breast and ovarian cancers and they are found at various locations in the genome.

Normal cells have protective factors that eliminate mutations in the genome and activate cell cycle checkpoints. Normal cells have lost highly specific molecules such as pRB, p53, and BRCA1/BRCA2 becoming cancer cells. The discovery of novel genes and/or proteins and understanding of their working mechanisms can help the scientists to understand the mechanism of tumorigenesis.

23.1.6 Mutations in cell cycle checkpoints

The cell cycle is a process by which a cell increases its contents by duplicating and then divides with certain regulatory proteins for cellular reproduction such as oncogenes, tumor suppressor genes, cyclin proteins, and cyclin-dependent

kinases and mitotic checkpoint proteins. In the cell cycle progression, the mitogenic signals stimulate the cyclin D and cyclin-dependent kinase (CDK) 4 and CDK6, thereby resulting in the progression from G1 phase into S phase, phosphorylating the target proteins such as pRB (Otto & Sicinski, 2017). In this regulation, the cyclin D1 gene (CCND1) is highly amplified in all cancer types (Beroukhim et al., 2010), while the introduction of CDK4 with a point mutation (R24C) into a mouse causes tumorigenesis in numerous tissues (Sotillo et al., 2001). Additionally, CDK4 with this point mutation, $NRAS^{Q61K}$ oncogenic mutation and RXRα loss in the epidermal keratinocytes cause the melanoma invasion through the lymph nodes (Chagani et al., 2017). Moreover, amplification of CDK4 amplicon in glioblastoma (GBM) contributes to the driving of the GBM tumorigenesis (Qi et al., 2017). R31C knock-in mutation increases the binding capacity of p16 (INK4a) which is an inhibitory protein of CDK4/6 to CDK4 in *Bcr-Abl* transformed hematopoietic cells. CDK4 and CDK6 work together in hematopoietic tumor progression and Cdk6 is recruited for preventing the interaction of CDK4 and INK4 proteins (Rodriguez-Diez et al., 2014).

During the late G1 phase, cyclin E increases CDK2 activity with E2F-mediated transcription (CCNE1) and then, cyclin E/CDK2 complex starts to phosphorylate the progression of cell cycle, centrosome duplication and DNA replication-related proteins (Otto & Sicinski, 2017). E2F-mediated transcription of cyclin E (CCNE1) is amplified in various malignancies, especially breast and ovarian cancers (Etemadmoghadam et al., 2013; Scaltriti et al., 2011). During S phase, cyclin E, which aids cells to entry into S phase with pRB phosphorylation is removed from the complex by the ubiquitination process (Davis, Swanger, Hughes, & Clurman, 2017; Whittaker, Mallinger, Workman, & Clarke, 2017). This ubiquitylation can be realized by Golgi-associated RhoBTB3 protein, thereby promoting the cell cycle progression for increasing the cell surface area (Lu & Pfeffer, 2013). CDK2 associates with cyclin A2 to construct cyclin A-CDK2 complex. This cyclin A-CDK2 complex is recruited to terminate the S phase of cell cycle (Whittaker et al., 2017). In recent years, there are significant articles that target the signaling pathway of cyclin A-CDK2 complex using computational analyses. First, sialic acid binding to CDK2 and aspirin downregulates this complex in colon, breast, and skin cancers, thereby decreasing cancer cell proliferation (Dachineni et al., 2016). Secondly, constructed inhibitors using computational analysis were synthesized to impair cyclin A/CDK2 interaction with CDC25B, a cell cycle regulator targeting CDC25B (Li, Ma, Ma, et al., 2017).

During the G2 phase, CDK1 that is crucial for cell cycle progression binds to cyclin B and cyclin A2. After a cell enters into mitosis, this complex reveals cyclin A2 by degrading and the activity of complex required for mitotic entry is sustained by cyclin B (Gavet & Pines, 2010). During interphase, CDK1-cyclin A2 complex stimulates the adhesion complex to interact with extracellular matrix and organization of actin cytoskeleton. This complex modulates the area increase of adhesion complex in the cellular transition through S phase (Jones, Askari, Humphries, & Humphries, 2018). In the late mitosis, cyclin B is degraded to become CDK1 fully active in the chromosome separation (Otto & Sicinski, 2017). Comparing to other CDKs in the coordination of S phase, CDK1 is dysregulated by genetically alterations during tumorigenesis (Asghar, Witkiewicz, Turner, & Knudsen, 2015). The ablation of CDK1 in liver tissue diminishes liver tumorigenesis (Diril et al., 2012) and the inhibition of CDK1 decreases the KRAS-mutant colorectal cancer mice models (Costa-Cabral et al., 2018). KCTD12, potassium channel tetramerization domain containing 12, is dramatically overexpressed in lung and cervical cancers and its expression is related to larger tumor areas and poor survival rates. The results showed that KCTD12 interacts with CDC25B and aids CDK1 phosphorylation to provide the G2/M transition, thereby resulting in tumorigenesis (Zhong et al., 2017).

Collectively, CDKs are important regulators of the cell cycle process in both normal and cancerous cells. The inhibition of CDKs might specifically kill tumor cells. However, the articles about the inhibition of CDKs required synthesis of novel inhibitors and their investigations on mouse models to develop our future biomedicine therapies.

23.1.7 Mutations in cellular growth-related genes or proteins

Normal or cancerous cells can uptake growth factors with upstream receptors such as EGFR as a signal for maintaining cell proliferation and preventing apoptosis. This signal is transmitted from these receptors to transcription factors via the Ras/Raf/MEK/ERK signaling cascade for regulation of gene expression as well as the PI3K/PTEN/Akt signaling pathway. Abnormal activation of these pathways has been demonstrated in various human cancers upon mutations at upstream receptors and Ras and Raf mediator proteins (McCubrey et al., 2007).

The data mining of PubMed-based literature about EGFR mutations reveals that exon 19 deletion has been documented in various cancer types such as ovarian, pancreatic, and nonsmall cell lung cancers as well as a point mutation in exons 19, 20, and 21 of NSCLC, Barrett's esophagus and prostate cancers (Chintala & Kurzrock, 2010). To eliminate the negative effects of these mutations on cancer patients, many novel drugs have been synthesized. One of these candidates, an EGFR-associated tyrosine kinase inhibitor, osimertinib, targets EGFRs with exon 19 deletion and/or exon 21

bearing point mutation L858R and resistance mutation (T790M) in NSCLC patients. Therefore osimertinib increases significantly the survival rate compared with other EGFR-TKIs (Soria et al., 2018). In addition to osimertinib, YH25448 is another third-generation EGFR-TKI to treat the similarly mutated NSCLC with higher selective efficiency. In vitro and in vivo results have indicated that this drug dramatically inhibits EGFRs bearing the same mutations by binding selectively and irreversibly and downstream signaling of EGFR compared with osimertinib (Yun et al., 2019).

Ras, a small GTP-binding protein (GTPase), is an upstream mediator of signaling pathways. There are three human RAS genes, KRAS, NRAS, and HRAs encoding four RAS proteins with two isoforms of KRAS: KRAS4A and KRAS4B. RAS genes with missense mutations (gain of function) have been documented in 27% of human cancers and 98% of these mutations occur in three hotspots (G12, G13, and Q61). It is considered that these mutated RAS proteins are suspended in GTP-bound level and GTP bound to RAS is not able to be hydrolyzed in cancer (Hobbs, Der, & Rossman, 2016). KRAS in colorectal and pancreatic cancer and NSCLC is the most seen (85%) Ras protein and regulates proliferation and survival activating downstream pathways (Hobbs et al., 2016, Kawada, Toda, & Sakai, 2017). KRAS mutations—G12D, G12V, and G12R—can be found in PDAC (pancreatic ductal adenocarcinoma) patients and G12D is related to poor overall survival. Thus G12D mutation in KRAS GTPase should be considered as a promising prognostic factor for advanced PDAC (Bournet et al., 2016). The KRAS G12V mutation and p53 deficiency may participate in NF-κB activation, thereby resulting in cell cycle dysregulation and apoptosis suppression. Thus these lung cells have undergone tumorigenesis (Yang et al., 2015).

Raf, a serine/threonine kinase is mainly activated by Ras interaction and transduces a signal through mitogen-activated protein kinase (MAPK). The family of the Raf gene has three mammalian members: A-Raf, B-Raf, and C-Raf (McCubrey et al., 2007). B-Raf (BRAF) is one of the most seen mutated kinases in cancer cases such as melanoma, thyroid, colorectal cancers, and NSCLC (Dankner, Rose, Rajkumar, Siegel, & Watson, 2018). BRAF can be mutated mainly at the V600 site changing to D/E/K/R amino acids in the polypeptide chain, thereby resulting in BRAF increased activity (Wan et al., 2004). MicroRNA-211 is a crucial regulatory oncogene of melanoma (Dror et al., 2016) and its loss results in decelerated cellular growth of BRAF mutant cells, inhibition of melanoma tumorigenesis, PI3K signaling inhibition, and in vivo melanoma growth inhibition. In the $BRAF^{V600E}$ mutant melanoma, vemurafenib, a BRAF targeted drug, is applied to the melanoma cells and miR-211 expression is increased in vemurafenib-resistant melanoma cells. The loss of miR-211 increases sensitivity of melanoma cells to vemurafenib treatment (Sahoo, Sahoo, Joshi, Lee, & Perera, 2019). Overall, the combinational treatment might increase the therapy chance of melanoma patients.

The mutations in cellular growth-related genes might cause tumorigenic activity of their proteins. General cellular growth signaling pathways have undergone mutations and then these mutations drive the tumorigenesis by transducing the signals from tumor microenvironment (TME) to transcription factors. The understanding of tumor-causing alterations in vitro and in vivo provides the development of personalized therapy in the new biomedicine approaches.

23.1.8 Epigenetic changes during tumorigenesis

All human cancers have experienced genetic changes and these changes are corroborated with epigenetic changes to drive cancerous cell phenotype. The epigenetic changes can be sourced by DNA methylation enzymes, histone-related proteins, chromatin remodelers, and microRNAs (Baylin & Jones, 2016; Pfister & Ashworth, 2017).

DNA methylation alterations on CpG islands contribute to tumorigenesis in three different ways: general hypomethylation (decreasing of methylation) of normal cell genome through cancer cell genome, hypermethylation (increasing of methylation) of tumor suppressor genes (TSGs), and methylation of cytosine (5mC) by some factors: deamination, carcinogens, and UV (Baylin & Jones, 2016). Deletion or inhibition of DNA methyltransferase that might add a methyl- group into specific sites of CpG islands causes an increase in transcription levels of transposable elements and oncogenes, which allow genome instability. Hypomethylation of LINE-1, long interspersed nuclear element-1, allows the activation of metastasis-related protooncogenes: MET, CHRM3, and RAB3IP, thereby resulting in the aggressive CRC metastasis progression (Hur et al., 2014).

Normal cells have become cancerous cells with mutations in the tumor-specific driver gene and TSGs. TSGs are predominantly hypermethylated in their promoter sites to suppress their gene expressions. DNMT1, DNA methyltransferase-1, is overexpressed in pancreatic cancer to hypermethylate the promoter of miR-148a that functions as a tumor suppressor miRNA. The upregulation of miR-148a inhibits DNMT1 activity by binding its 3'UTR and blocks the methylation of pancreatic cancer-related TSGs such as p16 and RASSF1A by decreasing DNMT1 activity. The overexpression of miR-148a in the pancreatic cancer cells causes a decrease in cell proliferation, epithelial mesenchymal transition (EMT), and invasion (Hong et al., 2018). Conversely, TET1 DNA demethylase that aids to remove

the methyl group from 5mC is overexpressed in triple-negative breast cancer (TNBC) tumors to activate the PI3K-mTOR pathway for oncogenic proliferation. The deletion of TET1 in TNBC cells reduces the cellular proliferation and PI3K related genes and higher expression of immune response-related genes (Good et al., 2018).

Histone proteins are able to wrap double-stranded DNA to construct nucleosomes, which form the secondary structure of DNA linear strands to regulate the DNA accessibility for cellular events (Pfister & Ashworth, 2017). The chromatin remodelers regulate nucleosomes' positioning and chromatin accessibility to allow transcription factors or DNA-binding proteins (Langst & Manelyte, 2015). AT-rich interactive domain-containing protein 1A (ARID1A) is a commonly mutated chromatin remodeler in ovarian clear cell carcinoma (OCCC). ARID1A might bind active regulatory elements such as enhancers and promoters in OCCC to increase transcriptional activities of over 200 genes. The depletion of ARID1A causes a decrease in the transcription of these because of the changes in chromatin accessibility. In the absence of ARID1A, this alteration causes the downregulation of RNAII polymerase-dependent genes. The downregulation of ARID1A causes the upregulation of ARID1B activity to transcribe the PRNAII associated genes in ovarian clear cell carcinoma (Trizzino et al., 2018).

The last group in the epigenetic regulation of tumorigenesis is histone-related proteins such as histone modifiers and readers (Baylin & Jones, 2016). These histone modifiers can modulate the chromatin function and structure by histone tail modifications such as acetylation, methylation, and phosphorylation (Pfister & Ashworth, 2017). YEATS2 gene coding a histone H3K27ac reader is highly overexpressed in NSCLC to modulate cell survival and tumor growth by binding with YEATS domain to acetylated histone H3. The colocalization of YEATS2 and H3K27 acetylation on promoters of transcribed genes stimulate the activation of their transcriptions in cancer cells. The depletion of the YEATS2 study has revealed that H3K9ac levels on the genes encoding ribosomal proteins are reduced and this causes the deactivation of essential genes in NSCLC tumorigenesis (Mi et al., 2017). Hepatocellular carcinoma (HCC) cells have a higher level of histone deacetylase 5 (HDAC5) compared to normal hepatic cells and HDAC5 knockdown attenuates the proliferation of cancer cells increasing the H3K9 acetylation at the promoter of TAp63 gene to stimulate its expression. The upregulation of TAp63 allows the inhibition of proliferation of HCC cells by stimulating the cell cycle arrest. Additionally, maspin that is a tumor suppressor protein is downregulated in many human cancers and regulated by p53 tumor suppressor. TAp63 modulates the maspin expression to inhibit the tumor growth of HCC cells. Collectively, higher expression of HDAC5 in HCC cells stimulates acetylation of histones at promoter site of TAp63 to inhibit the TAp63 and maspin transcription for HCC progression (Gu et al., 2018).

Although there are various reasons causing tumorigenesis in normal tissues, tumorigenesis can be caused by epigenetic changes in normal tissue. DNA methylation enzymes, chromatin remodelers, histone-associated proteins, and microRNAs have the ability to drive or cause the tumorigenesis in numerous cancer types. The revealing of mechanisms causing the tumorigenesis in healthy tissues aids the increasing the number of treatment methods in future personalized biomedicine.

23.2 Genetic profiling for genomic instability in cancer cells

It is reported by GLOBOCAN 2018 database that there were 18.1 million people diagnosed as a cancer patient and 9.6 million patients died because of cancer worldwide. Cancer is one of the major causes of death and 1 in 10 women and 1 in 8 men are going to die because of cancer. Of these cancer deaths, 18.4% (1.8 million patients) will be associated with lung cancer and thus lung cancer is a major cause of cancer mortality worldwide. Similarly, the estimated number of new cases in female breast cancer is approximately 2.1 million and about 600,000 women will die from breast cancer (Bray et al., 2018). The diagnosis of these leading causes of death during progression in the patients is crucial for the decrease in these cancer death statistics. Within this context, cancer cell-specific sequencing methods, whole genome sequencing, exome sequencing and targeted gene sequencing using DNA and mitochondrial DNA of cancer cells and tumor progression-related genes, RNAs and proteins and noninvasive cancer biomarkers (https://www.illumina.com/areas-of-interest/cancer/research/sequencing-methods.html) (Illumina, 2019b) will be examined in this section to understand the current methods to identify cells in tumorigenesis.

23.2.1 Cancer cell-specific (DNA and mitochondrial DNA) sequencing

Cancer is a heterogeneous disease that has unique phenotypic and genomic properties (Zhang, Spath, Marjani, Zhang, & Pan, 2018), thus there are no two cancer patients who have the same clinical pathway (Vogelstein et al., 2013). The determined ways for cancer treatments in clinics are transforming through personalized therapies containing individual patient information. This information includes individual genomes of cancer cells in the tumor mass, functional regions

for protein-coding, and regulation such as untranslated regions and identified variants of cancer cell-specific genes. The individual cancer cell genome can be obtained by whole−genome sequencing (WGS) approach using next-generation sequencing (NGS) method. This approach identifies nucleotide substitutions, repetitive element rearrangements, indel mutations, microbial infections, copy number changes, translocations, and inversions in the whole cancer genome compared with the normal reference genome. In this approach, WGS is highly functional to determine the breakpoints of inversions and translocations in the chromosome structure, especially in solid tumors, as well as finding novel mutations in the cancer genome resulting in tumorigenesis. Chromosome-related studies using the WGS method in two cell lines of lung cancer and one cell line of acute myeloid leukemia have uncovered the presence of mutations, chromosomal translocations, inversions, and fusions in 2008 compared with normal cell types of cancer types (Tuna & Amos, 2013).

One of the first studies using the WGS method has revealed that acute promyelocytic leukemia (APL) is related with the presence of a reciprocal fusion gene named as bcr3 *PML-RARA* fusion gene arising from t(15;17) translocations. This fusion gene is a cytogenetic invisible oncogene, which could not be detected by classical reverse transcription polymerase chain reaction (RT-PCR). Therefore a patient was treated with the classical acute myeloid leukemia (AML) agents and the patient was getting worse. The WGS study of her bone marrow cells revealed the presence of the oncogenic fusion gene resulting in APL instead of AML. After this discovery, the treatment pathway was changed and similar cases were examined using WGS method to catch *PML-PARA* fusion (Welch et al., 2011). With developing strategies such as WGS, a used treatment way can be altered based on the genetic profiling of the patient genome.

The recent studies have demonstrated the effectiveness of WGS in the determination of novel tools and methods in the most seen cancer types. As indicated in the above part, *BRCA* genes have essential functions to modulate the genome stability through HR-related DSB repair system. Since all advances in the detection and treatment of *BRCA* mutated breast cancer patients are not functional to understand the mechanism behind *BRCA* gene loss in cancer genome, DNA sequences of 560 breast cancer patients were analyzed with WGS method. After the classification of sequences based on the cell types with *BRCA1/2* deficiencies, HRDetect predictor can analyze and report the differences between germline *BRCA1/2* carriers and breast cancer samples. The differences are obtained in 20 common alleles, 107 rare variants, and eight missense mutations. Additionally, HRDetect can recognize *BRCA1/2* deficient tumors separately (Davies et al., 2017). Collectively, cancer cells have many mutated nucleotides in different areas but these mutations can be related with a few coding regions that inhibit, regulate, or induce tumorigenesis and resist widely used agents. These mutations have been demonstrated by the aid of machine learning ability, prediction tools, and WGS method.

Papillary thyroid carcinomas (PTCs) are known as painless tumor types but some patients with this disease have undergone distance metastasis causing patient death. The current treatment strategies have no reliable markers that are able to detect distant metastatic PTC cells. To find specific and reliable biomarkers related to metastatic PTC cells, DNA sequences of 49 DM PTCs and 97 control patients were analyzed using the WGS method. Results indicate Chr1q duplication, Chr5q duplication near TERT genomic locus, and TERT promoter mutations. These three differences are identified as THYT1 signature for a molecular prognostic marker in distant PTCs. The presence of this signature can be analyzed by preoperative fine needle aspiration biopsy (FNAB). After obtaining these analysis results, DM PTC patients with THYT1 signature have a sevenfold much higher risk when compared to THYT1-negative control patients (Gandolfi et al., 2018). Collectively, the diagnosis of DM PTCs was changed with the WGS method and this diagnosis using THYT1 signature can increase the overall survival.

In addition to the genomic DNA sequencing, the latest research articles include mitochondrial DNA (mtDNA) sequencing to analyze mtDNA contents in various cancer types using WGS and whole exome sequencing. An study indicates that mtDNA copy numbers of some cancer types are associated with expression levels of respiratory genes and mtDNA depletion is tolerated to maintain the respiratory proteins in the tumor cells. It was also documented that an increase in TP53 mutations is dramatically associated with the aberrant mtDNA activity (Reznik et al., 2016). The whole mitochondrial genome sequencing of pituitary adenomas also reveals that 414 novel variants were identified in these adenomas and the presence of a variant (T16189C) can be related to the benign behavior of human pituitary adenomas. Taken together, whole mitochondrial genome sequencing can be a reliable approach to diagnose pituitary adenomas based on their characteristic variants (Nemeth et al., 2019).

Whole exome sequencing (WES or WXS) is a specialized DNA sequencing approach that contains exome (whole-coding regions), noncoding DNA sequences in exon-flanking DNA parts such as microRNAs, specialized promoter regions and untranslated regions (UTRs). The coding exons sequencing is crucial to discover the somatic mutations from specialized gene families to the cancer types with low cost and time saving to detect less cancer specific mutations (Tuna & Amos, 2013). WES has a limitation to detect the roles of noncoding regions in the genome compared to WGS. However, WGS studies have documented that more than 80% of tumor-specific variants might be located in noncoding

regions (Manolio et al., 2009). WES studies are potentially increasing in various diseases and cancer to detect cancer or disease-specific variants. One of these cancer-specific studies is about ovarian adult granulosa cell tumor (aGCT), which is most seen malignant ovarian tumor types. This study has demonstrated in 22 patients using WES that aGCT can be characterized by a somatic mutation of *FOXL2 p.C134W* forkhead transcription factor. This study also used targeted gene sequencing to identify the promoter sequence variants of the *TERT* gene, which is a coding region for telomerase catalytic subunit for more reliable results. The analysis results indicate that exome and targeted sequences of recurrent aGCT patients have *FOXL2* mutation with -124C > T *TERT* promoter mutation. Additionally, it was documented that these recurrent patient samples have developed metastasis. The presence and/or absence of this mutation especially *TERT* mutation after detection of *FOXL2* can indicate the metastasis ability of the tumor samples (Alexiadis et al., 2019). WES is another sequencing approach to identify the differences in exon and exon-associated sequences of tumor samples and is a promising approach to generalize personal medicine in future clinics.

Collectively, the development of leading technologies has revolutionized in both wet laboratory and clinical studies to understand the cancer cell genome. WGS and WES might permit researchers to detect disease-associated variants, inheritance mechanism of these variants, genetically meaningful alterations in somatic cells through tumorigenesis, invasion, and metastasis. Thereby, the alterations and variants have improved tumor diagnosis, classification, and prognosis for determination of which specialized therapy with disease-stage specific biomarkers for a patient as future biomedicine.

23.2.2 Genetic tests for tumor progression related genes, RNAs or proteins

Cancerous cells in tumor mass can differentiate by mutating genes and their RNA and protein variants, altering gene expression levels, other RNA levels recruited for mRNA translation or regulation of transcription and proteins, which affect cellular events from survival through apoptosis. Additionally, these cancer cells can lose their position in tumor mass, their cytoplasm and nucleus (DNA, mtDNA, and RNAs are becoming free) or other molecules such as proteins and exosomes, thereby leading to circulation in the blood.

Several RNA types as discussed in the above episode can modulate tumor progression. Whole-transcriptome sequencing (RNA-Seq) is a leading approach to understand roles of mRNA, miRNA, and other RNAs in tumorigenesis, invasion, and metastasis. RNA-seq might be used after RNA/cDNA conversion to discover and identify novel tumor-specific splice variants; to classify and quantify the tumor-specific transcripts of cells at various tumor progression stages in and out of tumor mass; to define expression levels of allele-specific transcripts; and to reveal a modulation of transcription and RNA biogenesis in tumorigenesis (Tuna & Amos, 2013). Total RNA is isolated and converted into cDNA to reveal mutated sequences in RNA-seq related cancer studies. One of these studies about clear cell renal cell carcinoma (ccRCC) uncovered 290 differentially expressed genes obtained from RNA-seq data of 33 patients and The Cancer Genome Atlas (TCGA). Higher expression levels of four genes: G6PD, GCNT3, APLP1, and PLPP2, are strongly associated with advanced stages of ccRCC and PLPP2 and APLP1 upregulation have exhibited poor overall survival. This RNA-seq of patient and TCGA data identified novel ccRCC biomarkers to diagnose diseases and this transcriptomic study may revolutionize treatment methods of diseases (Batai et al., 2018). Another RNA-seq method using a xenograft model of TNBC also demonstrated that an antitumorigenic molecule, melatonin, can significantly modulate expression levels of Tnfaip8l2 and Il1f6, thereby suppressing tumor growth. The melatonin upregulation analyzed by RNA-seq transcriptomic regulates TME promoting CD8 + T and NK cells and secreting tumor suppression-mediated cytokines. Collectively, melatonin treatment upregulates immune response genes to suppress the growth of tumor cells and activate immune system cells (Jardim-Perassi et al., 2019).

Targeted cancer gene sequencing is another next-generation sequencing method that includes only specific sequencing of tumor-associated genes such as exons of interested genes to detect even less frequently seen mutations. In addition to detection of cancer gene sequencing, the targeted gene sequencing approach might detect inherited mutations of cancer patients, alterations in tumor progression, and noninvasive biomarkers. Targeted gene sequencing provides several advantages because of smaller sequenced regions, thereby resulting in it being less money- and time-consuming (https://www.illumina.com/techniques/sequencing/dna-sequencing/targeted-resequencing/targeted-panels.html) (Illumina, 2019a). A recent study with targeted sequencing of *BRCA1* and *BRCA2* genes after isolation of DNAs from blood samples has revealed that two novel mutated variants of *BRCA1* and *BRCA2* tumor suppressor genes are clearly related to contracting breast cancer in a Russian population. This study also indicates that the presence of these variants might be used as promising biomarkers to diagnose familial breast cancer. This type of analysis that reveals specific variants to individuals or specialized groups or families may contribute to the development of personalized therapies in biomedicine (Solodskikh et al., 2019).

A liquid biopsy is a kind of approach for analyzing nonsolid biological materials to diagnose diseases. In this approach, noninvasive cancer biomarkers are circulating tumor cells (CTCs), circulating tumor DNAs (ctDNAs), circulating mitochondrial tumor (cmtDNAs), circulating RNAs, proteins, and exosomes (Afrifa, Zhao, & Yu, 2018; Han, Wang, & Sun, 2017). CtDNAs are promising biomarkers that can be detected easily in the first stages of cancer for personalized therapy in the future age of biomedicine. The studies have demonstrated that ctDNAs are believed to be highly specific tumor-derived DNA fragments from apoptotic or necrotic cancerous cells (Snyder, Kircher, Hill, Daza, & Shendure, 2016). The ctDNA-based sequencing is highly sensitive to visualize tumor progression as well as drug resistance (Shu et al., 2017). CtDNAs have approximately 167 bp and studies are highly focused to classify the tumor-specific mutations using targeted sequencing (Snyder et al., 2016). One of the recent studies comparing ctDNAS to tumor DNAs of 58 NSCLC patients has identified 50 mutated genes and four of them are the driver somatic gene mutations: *EGFR*, *PIK3CA*, *KRAS*, and *TP53*. These genes are also correlated with the tumor aggressiveness. The presence of these mutated genes and their products might inform a clinician to make a highly possible prediction about tumor progression (Chen et al., 2016). The very similar study containing targeted sequences of seven genes: *BRAF*, *ERBB2*, *PIK3CA*, *EGFR*, *PTEN*, *KRAS*, and *NRAS* in 50 patient's blood samples has documented that the *EGFR* gene frequently has a mutated protein at T790M point in NSCLC patients (Sim et al., 2018). In addition to ctDNAs, cmtDNAs are smaller than ctDNAs and their sizes are approximately 16 kb. Higher mtDNA copy number allows easier detection in the liquid biopsies of the patients. Tumor-specific cmtDNA studies have documented that the levels of cmtDNAs in the blood samples of aggressive and/or metastatic stage cancers are much higher compared to the first stages of these cancer types such as bladder, prostate and renal cancers (Afrifa et al., 2018; Ellinger et al., 2012). The presence and increased levels of cmtDNAs provide information about the tumor stage, lymph node, and hormonal receptor statuses in breast cancer patients (Afrifa et al., 2018; Mahmoud, Fawzy, Ahmad, & Ali, 2015).

Liquid biopsy also reveals the proteins in the blood circulation to be noninvasive biomarkers that are related to tumor progression. Prostate specific antigen (PSA) is the most used noninvasive biomarker for prostate cancer, which is the second cause of cancer death in males (Liu et al., 2017). The presence of total PSA in the blood (ng/mL) is not sufficient to visualize the tumor progression. Thus PSA density ($ng/mL^2 \times 100$)-related study has documented that increased PSA density in the circulation is associated with advanced stages of tumors. PSA density can be calculated by dividing PSA in nanogram/milliliter into the prostate volume as milliliters. The determined average PSA density values are crucial for biopsy decisions and tumor progression (Nordstrom, Akre, Aly, Gronberg, & Eklund, 2018). The regulation of PSA in the prostate cancer (PCa) cells is provided by FOXM1 which is a highly expressed proliferation-associated transcription factor in PCa cells. FOXM1 transcription factor specifically binds promoter/enhancer regions of PSA to induce tumorigenesis in prostate epithelial cells. The depletion of FOXM1 gives rise to the inhibition of PSA transcription and thus FOXM1 might be a therapeutic target with PSA protein in the blood circulation to visualize the prostate cancer progression (Liu et al., 2017).

The progression through tumorigenesis is modulated by mutated genes, RNA and protein variants, and altered transcription and translation processes. Sequencing of these molecules by the NGS method can uncover their roles or mechanisms in tumor progression. The diagnosis of the alterations using their blood samples might rescue patients from death or poor life quality. The development of a genetic test provides the tumor-specific genetic profiling of individuals and thereby results in improved personalized therapy for biomedicine in the near future.

23.3 Personalized therapies

Personalized medicine is an approach that examines a cause(s) of a disease and seeks solutions to the disease investigating the history behind the disease, interactions between te genetic background, lifestyle, and environment, and what therapies can be effective for patient survival quality. Personalized cancer medicine seeks an individual genetic background and tumor progression until the apoptosis of cancer cells. Within this context, clinicians and molecular biologists hope to reveal the most effective strategy to a patient to screen, prevent, and abolish the cancer cells. Genetic profiling of cancerous and normal cells using sequencing methods of whole genome and exome and tumor-related RNAs, genes, and proteins has demonstrated better individual treatment ways to patients with their needs. The major treatment pathway of personalized medicine is to identify the root cause of developing cancer in a patient and visualize the progression of the tumor; to carry out the best treatment option without any side effects and to eliminate the recurrence risk of cancer. Up to now, the alterations through the cancerous cells and detection methods using genomic profiling of cancerous cells in the tumor mass and blood circulation were examined to bridge an interaction between the molecular biologists and clinicians for the better treatment plan. This section will indicate current personalized

medicine strategies: genetic-based drug implementations, tumor-specific gene, and stem cell therapies, following circulating tumor cells and therapies and marker-based drug therapies developed by bioinformatic tools and immunotherapies.

23.3.1 Drug implementation based on the genetic background (pharmacogenomics)

Normal cells might generate various mutated genes and their variants to drive tumorigenesis in the tissue. The drugs used in the treatment of patients are specialized for known mutated genes. However, cancer patients might have more than one variant of mutated genes in cancerous cells in the current age. The standard genetic testing focuses on the specialized genes for a specific cancer type. With developments of biomedicine to find a better solution for a patient, clinicians and scientists seek other variants that give rise to tumorigenesis, multidrug resistance, and drug attrition in a patient using NGS methods.

Pharmacogenomics is a genetic profiling approach in cancerous cells of a tumor mass to aid the researchers to identify the interactions between unknown variants in their genomes and the antitumorigenic drugs. Even if personalized medicine is being common and the cost for the diagnosis of cancer disease is reduced, scientists should identify variations and reveal vital biomarkers that give rise the undesired drug responses including the drug inactivation or hyperactivity of the drug (Kranzler, Smith, Schnoll, Moustafa, & Greenstreet-Akman, 2017). The determination of this type of differences in individual patients helps clinicians to alter treatment methods to choose the most effective drugs, doses, and time intervals for patients. An exome sequencing-based study about drug-related genes in human has demonstrated that many genetic variants of drug-related genes may not be detected in clinical studies since they are significantly rare. The analysis of drug targets in cancer cells has documented that the genomic contents of the patients with germline variants can affect the responses of tumor cells to widely used cytotoxic agents, thereby altering the drug efficacy in patients (Scharfe, Tremmel, Schwab, Kohlbacher, & Marks, 2017). Determination of these changes using exome sequence analysis can provide a better approach to treat patients directly.

The glucocorticoid (GC) treatment response in acute lymphoblastic leukemia (ALL) provides the crucial biomarker of prognosis of the disease. The variants of pharmacogenomics-related genes NR3C1; glucocorticoid receptor gene, GSTs; glutathione S-transferase gene and ABCB1; multidrug resistance 1 gene encoding P-glycoprotein membrane transporter may contribute to GC response throughout the personalized GC therapy for ALL patients. The analysis of obtained DNA from blood samples of childhood ALL patients reveals that NR3C1 rs6198 variant and *GSTP1* rs1695-rs1138272 haplotype can be leading pharmacogenomic markers in ALL patients through the GC response (Gasic et al., 2018). The increasing similar studies will provide more reliable and promising genetic markers for successful personalized medicine.

In addition to somatic mutations, the investigation of germline variants in cancer cells are also crucial for pharmacogenomics and the most studies of germline variant for cancer drugs are on the drug metabolizing enzyme, cytochrome P450 2D6 (CYP2D6), that regulates the conversion of tamoxifen to N-desmethyl-tamoxifen and then subsequently to endoxifen. These metabolites are highly effective compared to tamoxifen in suppressing the estrogen-related cellular proliferation of estrogen receptor (ER)-positive breast cancer cells. CYP2D6 genetic variants may affect their enzymatic activities positively or negatively, thereby resulting in failure or success in the tamoxifen treatment. Thus CYP2D6 variants modulate the treatment decisions of tamoxifen for the survival of ER-positive nonmetastatic breast cancer patients (Drogemoller et al., 2019). A study about CYP2D6 genetic variants in 960 women, which can inhibit the conversion of tamoxifen to metabolites, thereby decreasing the efficiency of tamoxifen treatment, declared that the use of inhibitors against these CYP2D6 variants with tamoxifen treatment is not associated with the recurrence of breast cancer and/or breast cancer mortality. This article states that this combinational therapy is not functional in the increase in the tamoxifen efficiency and there are additional functional enzymes recruited in tamoxifen metabolism in ER-positive breast cancer patients (Mayer et al., 2019). The increasing pharmacogenetic studies in tamoxifen metabolism with CYP2D6 variants will reveal CYP2D6 polymorphisms-related enzymes, which can influence the tamoxifen metabolism by directly regulating the metabolic pathway and transporting tamoxifen (Cronin-Fenton & Damkier, 2018).

A novel pharmacogenomic study in epithelial ovarian cancer cells reveals for the first time roles of Neuregulin 3 (NRG3) rs1649942 and BRE rs7572644 that are identified by genome-wide association studies (GWAS). The patients bearing NRG3 rs1649942 A allele can live longer compared to other patients in tumor stage IV which is determined by the International Federation of Gynecology and Obstetrics (FIGO) following the Rustin criteria. Conversely, patients with the BRE rs7572644 C allele present predominantly decreased overall survival compared to TT-homozygous patients, who carry the dominant traits of the gene of interest (Pinto et al., 2019).

Cancerous cells can have various variants related to the tumorigenesis, drug resistance, and drug metabolism in the organism. The activities of developed drugs can be altered by these variants in cancerous cells. Pharmacogenomics is a genomic profiling approach which examines the regulation of drugs in the metabolisms of cancer cells or patients. The developing approaches to profiling of individual cancer patients will increase the overall survival and life qualities of patients by identifying the variants of altered genes or proteins in the patients.

23.3.2 Marker-based drug therapies using wet lab techniques and bioinformatic tools

Bioinformatics is an emerging multidisciplinary science involving computer science, statistics, and biology. It includes the computational modulation and examination of biological input about genes to medical information and applications such as robotic surgery with lower cost, time, and higher processing of limitless data. Nowadays, single cell analyses have demonstrated the molecular biology separating the cancerous cells from the single healthy tissues, which supports the hypothesis of cancer stem cells in the tumor mass. The altered molecules in the cancer cells can be accepted as biomarkers for the bioinformatic analysis to find the best drug therapies from novel biomarkers for drug discovery and drug application in biomedicine.

The alterations in cancerous cells discussed in the first part of this chapter and the genomic profiling approaches of their differences in the genomic level discussed in the second part of this chapter might provide molecular biologists and clinicians with an understanding of the differences between cancerous cells and normal cells and allow them to find better personalized therapies in biomedicine. The genomic differences in the cancer cells found by genomic profiling approaches can be analyzed via the machine-learning intelligence of bioinformatic tools. The machine-learning algorithms can comprise the transcriptome sequencing data to seek novel differences and therapeutic targets in personalized therapies in biomedicine. This research group has used the gene expression data in soft tissue sarcomas from the Genotype-Tissue Expression (GTEx) project and The Cancer Genome Atlas (TCGA). The rare soft tissue sarcomas, which are difficult to diagnose for the cancer type and then treat, include leiomyosarcoma; smooth muscle tumor, liposarcoma; fat cancerous cells and undifferentiated pleomorphic sarcoma; and fibrosarcoma. This group compared gene expression profiles of the soft tissue sarcomas from TCGA and nonmalignant tissues from the GTEx project using an unsupervised algorithm, principal component analysis (PCA), and the deep neural network, TensorFlow (v1.6). Based on the outcomes, they revealed undefined diagnostic markers using random forest algorithms. Then, they classified the soft tissue sarcomas as three tumor subtypes using t-distributed stochastic neighbor embedding (t-SNE) algorithm. The machine-learning random forest approach was used to identify the prognostic genes in these subtypes comparing with data from the French Sarcoma Group. These identified prognostic genes were analyzed with the k-nearest neighbor algorithm to demonstrate that they can be accepted as predictive of the metastasis-free interval. The machine-learning analyses have documented that HMMR is a strong prognostic gene for disease-free interval and metastasis-free interval in leiomyosarcoma (LMS). The progress of HMMR was visualized by immunohistochemistry and the highly expressed HMMR is related to the poor outcome in LMS tissues. This protein constructs a complex with BRCA1/BRCA2 and in the absence of BRCAs, it is overexpressed to compensate for the deficiency of these proteins (van et al., 2019).

Up to now, this research group has identified the prognostic gene as a marker in a soft tissue cancer type using various machine-learning algorithms and wet-laboratory techniques. At this point, the novel treatment ways were identified by analyzing the differential expression of molecules in the regulation network. The transcription factors and their regulators, kinases in this network were analyzed using data from a database, ChIP-seq Enrichment Analysis (ChEA) and kinase enrichment analysis. The results of this analysis, which were combined with a connectivity map for identification of the best drugs for the tumor subtypes, revealed that the drugs doxorubicin and trichostatin A (TSA), histone deacetylases (HDAC) inhibitors, could be used as a stronger treatment for soft tissue sarcoma subtypes. The cell viability assays of TSA on soft tissue sarcomas have proved that TSA has an ability to eliminate cancerous cells. Further cell viability studies using other HDAC inhibitors showed quisinostat can be a more powerful agent to kill the soft tissue cancerous cells, especially LMS cells (van et al., 2019).

To summarize, machine-learning algorithms may reveal diagnostic biomarkers, genes encoding the prognosis of the cancerous cells and novel therapeutic ways for carcinomas such as soft tissue sarcomas based on the gene expression data. Thereby the improvements in the machine-learning algorithms may help clinicians and molecular biologists to understand the rare differences in the patient genomes for upcoming personalized medicine in the next decades.

23.3.3 Following circulating tumor cells and therapies

Circulating tumor cells (CTCs) are tumor cells that have the abilities to detach from the primary tumor mass and enter blood circulation while evading immune system cells. These CTCs relocalize in distant primary lesions and cause

metastasis in distant organs. The detection of CTCs is highly difficult due to their low frequencies in peripheral blood. CTCs are currently detected based on their highly expressed tumor markers such as epithelial cell adhesion molecules (EpCAM), MUC-1, cytokeratin (CK), and tumor-associated glycoprotein-12 (TAG-12) using antibody−antigen interaction. These molecules improve the detection techniques such as the CellSearch system that uses EpCAM antigens highly expressed by CTCs (Cai, Cen, Cai, Falar Luis, & Biskup, 2019).

CTCs have promising advantages to monitor alterations during the disease progression. Therefore improvements are highly important to detect the CTCs in patient blood accurately for the efficacy of personalized medicine. One of these improvements involves a novel surface marker, *O*-glycan sialyl-Tn (STn), a highly expressed membrane protein in advanced colorectal and bladder cancer without expression in blood cells. Altered glycosylation of surface proteins in cancerous cells is the distinctive property as discussed above. This article proves that overexpressed STn in the bladder and colorectal cancer CTCs can be a potential marker compared to EpCAM-specific CellSearch system to detect these CTCs using microfluidic devices. This device for colorectal cancer revealed that STn + CTC percentage is three times higher than the percentage of EpCAM + CTCs (Neves et al., 2019). This data indicates the importance of the discovery of correct markers for cancer progression in personalized treatments.

Discovery of specific driver mutations and surface markers using genomic profiling techniques will improve the detection of specialized CTCs in advanced cancer cases and rare diseases with poor prognosis. A case report about Ewing sarcoma in a patient revealed that after completed treatment, the patient had lymph node metastasis and EpCAM-positive CTCs of cervical origin confirmed by liquid biopsy. The genomic DNA sequences of CTCs revealed that there are some alterations in the CTC genome such as FGFR4, fibroblast growth factor receptor 4, that are the same with paraffin embedded Ewing sarcoma tissue. This alteration indicates that this mutation is identified as a germline mutation and CTCs are evolved from primary tumor cells (Lee, Lim, & Cho, 2018). Therefore monitoring of these CTCs during cancer prognosis may contribute to determining treatment strategies in future biomedicine for an individual.

Similar to every cancerous cell, CTCs have a highly expressed "don't eat me" signal named CD47 and a "don't find me" signal named CD274 or mostly known programmed death ligand, PD-L1. The blocking process of both surface molecules on CTCs that cause lung metastasis in 4T1 mouse tumor revealed that anti-CD47 and anti-CD274 treatment suppress the tumor growth in the lung tissue compared to control and single CD47 or CD274 antibody applications. Moreover, the blockade of both surface molecules enhanced the activation of cytotoxic T cells and natural killer (NK) cells to kill CTCs in vivo. This study shed lights on the CTC treatment using antibodies for dual-immune checkpoints on CTCs. Therefore combinational studies using these immune checkpoint antibodies and adoptive immune cells such as CAR-NK and CAR-T cells will achieve the removal of cancer cells for personalized medicine (Lian et al., 2019).

In addition to the preclinical studies, the progression of prostate cancer is mostly related to the mTOR pathway and angiogenesis through VEGF. The drugs used, bevacizumab, humanized immunoglobulin G1 antibody for VEGF inhibition, and an mTOR inhibitor temsirolimus, stimulated the removal of CTCs in the blood. However, this combinational phase study did not provide any clinical benefits for the patients and there was no correlation with PSA (Barata et al., 2018). There are several phase studies, which examine the interactions of these types of drugs with CTCs and the effects of CTCs on recurrence of the disease after several years. However, these studies are not sufficient to determine the correct strategies to treat the patients or eradicate the cancerous cells in the patient's blood. The development of genomic profiling of CTCs will provide the determination of alterations in CTCs compared to primary tumor cells and noncancerous adjacent cells.

23.3.4 Tumor-specific gene therapies

Cancerous cells have developed many changes in their genomes to propagate without any controls and to evade the immune regulators. The cancerous cell propagation-related studies target the suppression of driver genes, which predominantly enhances the proliferation of cancerous cells. Gene therapies are described as specific gene transfer to eliminate cells or malfunctioning genes. Somatic gene therapies for cancerous cells mainly derived from somatic cells include inhibition of tumor-causing gene activities directly/indirectly, augmentation of genes that suppress the tumor growth or function normally and killing cancerous or tumor-causing cells.

Gene therapy studies have been conducted directly using reprogrammed viruses, bacterial vectors, and nonviral agents, DNA-wrapped nanoparticles using lipids, polymers, and inorganics. Additionally, gene therapy studies contain RNA interference using shRNAs or siRNAs and epigenetic regulation of target genes using microRNAs (Bottai, Truffi, Corsi, & Santarpia, 2017). Viral vectors provide a better efficiency of modified gene transfer since viruses attach to cancerous cells and transfer the gene of interest into the cancerous cell genome. After approval of a tumor virotherapy

drug for personalized medicine, oncolytic viruses have been improved to kill the cancerous cells directly/indirectly. A study on adenoviral vector containing granulocyte macrophage-colony stimulating factor (GM-CSF) and cytosine deaminase (CD) as a suicide gene that converts 5-FU to 5-FC revealed that infected tumor cells enhanced antitumor activities of cytotoxic CD8 + T cells and decreased the levels of immunosuppressive cytokines in TME in vivo. Indeed, this adenoviral vector dramatically increases BALB/c mice survival compared to the nontreated and only CD-containing groups without any side effects (Akbulut, Coleri, Sahin, Tang, & Icli, 2019). The specific infection of oncolytic viruses increases the efficiency of tumor virotherapy for personalized biomedicine. Moreover, the immunostimulatory effect of oncolytic viruses may increase the combinational studies using adoptive immune therapies.

Another gene therapy related to tumor study includes the bacterial vector expressing the epidermal growth factor receptor variant III (EGFRvIII) for the treatment of squamous cell carcinoma. This EGFRvIII contains tumor-specific in-frame deletion of EGFR exons 2−7 that causes ligand-independent activity of the receptor with a 21-amino acid peptide for cancer progression. This peptide is placed into Gram-positive facultative intracellular nonpathogenic bacterium *Listeria monocytogenes* that is engineered to limit its harmful effects to hepatocytes in liver tissue for a cancer vaccine. This vaccine candidate highly expressed this altered protein in vivo and this protein enhances antigen-presenting cells maturation and EGFRvIII-targeting CD8 + T cell activation to remove squamous carcinoma cells. The microbe-related cancer vaccine exhibited promising results in vivo, eliminating the EGFRvIII-expressing cancerous cells (Zebertavage et al., 2019) and similar studies will provide a better efficacy of the microbe-based cancer vaccines for personalized biomedicine.

In addition to the gene therapies, stem cells can be used for the treatment of various cancers especially using or transplanting hematopoietic stem cells into the patients. This type of therapy is used to increase the number of functional cells adjacent to cancerous cells. Therefore the negative effects of cancerous cells on the immune response and immune-suppressive cells in TME can be limited to increase the efficiency of other treatment ways such as chemotherapy, radiotherapy, or adoptive immune therapies. However, this stem cell therapy has unanswered questions and several side effects on patient life quality and survival. One of these questions is about stem cell differentiation that has no certain explanation about development through specific functional cells with sufficient numbers to eliminate the cancerous cells in TME. Another challenge is the elimination of immune rejection of the transplanted cells into TME and regular function of these cells (Chivu-Economescu & Rubach, 2017). Similarly, mesenchymal stem cells (MSCs) can be transplanted into the hematologic malignancies but the studies documented conflicting results about their roles in the transplanted area: MSCs can enhance tumor progression in hematological malignancies by inhibiting the apoptosis of cancerous cells or MSCs can exhibit antitumor functions by eliminating tumor-inductive effects or arresting cells in the cellular division (Lee et al., 2019).

Gene therapies for specific tumors have been developed to eradicate the cancerous cells efficiently using numerous vectors such as oncolytic viruses, bacteria, and various nanoparticles. Their efficiencies are developed to target only cancerous cells in TME.

23.3.5 Immunotherapy using immune system cells

Personalized medicine is most suitable for cancer treatment that arises from genomic differences in cancer stem cells and cancerous cells. Adoptive cell therapy (ACT) is personalized medicine for cancer patients, which includes the recruitment of immune cells with anticancer function in cancer patients. The ACT is the early treatment model that uses tumor-reactive lymphocytes, which comprise naturally to fight the cancerous cells for cancer regression in the patients. These lymphocytes are obtained from patients and are grown under in vitro conditions. After selection of lymphocytes based on their tumor-fighting activity, they are applied to the host to modulate cancer progression. However, the selection of these lymphocytes is the limiting factor to best treatment in cancer progression because of their abilities to identify cancerous cells (Rosenberg & Restifo, 2015). Therefore clinicians and molecular biologist have revolutionized adoptive lymphocyte transfer to the host by transfecting genes encoding receptors specialized for the specific antigens to the host T-cells (Morgan et al., 2006). Genetically engineered T-cells are a highly promising player to recognize and to eliminate the cancer cells effectively while patients, clinicians and molecular biologists are fighting with cancer. Indeed, CAR-NK cells and macrophages are additional effector players in cancer immunotherapy.

23.3.5.1 CAR-T cells

Chimeric Antigen Receptor (CAR)-T cells are engineered T lymphocytes are programmed cells to kill the cancerous cells precisely through the recognition of the tumor antigens. The first CAR-T cells comprised an extracellular binding

domain, a transmembrane domain, and at least one intracellular signaling domain. The second-generation CAR-T cells have also a costimulatory domain to increase T cell proliferation in a host (Hartmann, Schussler-Lenz, Bondanza, & Buchholz, 2017). These CAR-T cells developed by Porter and his colleagues directly target surface antigen CD-19 bearing malignant B-cells in cancer patients. These CAR-T cells also contain costimulator CD137 receptor domain and signal-transduction CD3-zeta component for T cell activation. The application of CAR-T cells has resulted in the elimination of CLL in the patient bone marrow (Porter, Levine, Kalos, & Bagg, 2011). The third generation CAR-T cells includes more than one costimulatory domains for cytokine secretion and increasing proliferation of engineered T cells. The fourth generation CAR-T cells are developed by combining costimulatory domains, cytokines, and enzymes, which can degrade the extracellular matrix structure of solid tumors based on the properties of second-generation CAR-T cells to promote the antitumor activities of CAR-T-cells (Hartmann et al., 2017). Unlike these developments in CAR-T cell-based therapies, CAR-T cell therapies have promising results against B-cell carcinomas due to higher expression of CD19 and significant selection of this biomarker by CAR-T cells in B-cell malignancies. The patients can tolerate the loss of CD19-positive noncancerous cells (Lim & June, 2017), however, cytokine release syndrome is the challenging problem as in the case of the first child who received the CD19 receptor bearing CAR-T cells (Rosenbaum, 2017). The improvement in the CAR-T cells provides the novel candidates for target antigens in cancerous cells. For instance, combinational receptor application for CD19 and CD22 antigens provides an increase in the remission in relapsed B-ALL (Huang et al., 2017) and the combinational application with this type of CAR-T cells and hematopoietic stem cell transplantation (HSCT) after infusion of CAR-T cells to the patient has been demonstrated to be the better option for high-risk B-cell lymphoma patients (Cao et al., 2018). Besides improvements in CAR-T cells for personalized medicine using individual T-cells as a treatment method of ACT, the US Food and Drug Administration (FDA) has approved CAR-T cells therapies: Kymriah (tisagenlecleucel) and Yescarta (axicabtagene ciloleucel) for the treatment of B-cell precursor ALL and large B cell lymphoma in adults respectively (US Food & Drug Administration, 2018, March 26; Administration, 2018, March 3).

Although CD19 targeting CAR-T cell therapies have demonstrated the significant successes in hematological malignancies, CAR-T cell therapies in solid tumors have overcome major solid tumor characteristic challenges such as matrix barriers and abnormal vascular pattern (Yan & Liu, 2019). The researchers seek different antigens to eliminate solid tumors directly. One of these studies includes EGFRvIII, expressed only in the glioblastoma (GBM) and 31%−64% of GBM patients have contained EGFRvIII for higher tumor cell proliferation and angiogenesis, thereby resulting in the higher mortality of the patients. This study documented that EGFRvIII-targeting CAR-T cells can suppress the proliferation of EGFRvIII-positive GL261 cells in vitro and in vivo. Higher dose application of these CAR-T cells to C57BL/6 mice has removed xenograft tumors clearly. Indeed, immunohistochemistry staining of xenograft tumor has shown that CD8 + T cells were significantly found in antigen-expressing tumors after infusion of EGFRvIII-targeting CAR-T cells to the mice. Moreover, an infusion of EGFRvIII-targeting CAR-T cells can achieve the elimination of EGFRvIII-negative GL261-cells efficiently (Chen, Sun, et al., 2019). Developments in solid tumor-specific CAR-T cells can increase the therapeutic activities of adoptive T cell therapies.

Although CAR-T cell-related studies have promising results for patients who experience the worst conditions during treatments, this treatment method has also various problems for modern biomedicine to prolong the overall survival and life qualities of patients who have hematological and solid carcinomas. The first problem is the infiltration and trafficking of CAR-T cells in the body circulation and solid TME. The infiltration activity of CAR-T cells into solid tumor region is the significant problem for the achievement of a treatment. The second problem is maintaining persistence and proliferation of CAR-T cells in the blood circulation to eliminate cancerous cells successfully in both hematological malignancies and solid tumors. The third problem is the recognition of cancerous cells by CAR-T cells via their antigen receptors. The noncancerous cells also include these antigens on their surfaces. This problem, called the on-target/off-tumor effect of CAR-T cells, can be managed with the combination of other surface antigens related to recognition and activation of CAR-T cells for killing cancerous cells while engineering CAR-T cells to eliminate cancerous cells, especially solid tumors. Another problem is the reorganization of TME in both solid and hematological malignancies. Although CAR-T cells are highly active to recognize and to kill cancerous cells, these cancer cells can deactivate CAR-T cells via PD-1 or CTLA-4 mediated interactions or numerous immunosuppressive molecules. The final problem is controlling CAR-T cells in in vivo studies to inhibit the toxic effect of CAR-T cells in the body (Martinez & Moon, 2019; Ye et al., 2018). The improvement in switch mechanisms of CAR-T cells sheds light on the control of CAR-T cells, such as using caspase-9 apoptotic protein for safe personalized cancer immunotherapy (Martinez & Moon, 2019; Wang, Chang, et al., 2011; Zhou & Brenner, 2016).

In addition to the improvements and challenging problems, researchers and clinicians have revolutionized multieffectors combinational therapies for high-risk B cell precursor ALL patients. In 2017 the FDA approved blinatumomab,

which is a modulator of an interaction between CD3-bearing T cells and CD19-bearing precursor B cells of ALL. Blinatumomab can specifically be identified as a BiTE, bispecific T cell engager that provides T cell recognition of malignant B cells in refractory and relapsed (R/R) cases of precursor B cell ALL. Both TOWER and ALCANTARA clinical trials have demonstrated that Philadelphia chromosome (Ph)-negative and Ph-positive R/R B-ALL patients have lived longer and experienced fewer side effects of any treatments, respectively, compared to chemotherapy and HSCT (Pulte et al., 2018). Based on these safety advantages of BiTE, researchers had decided to use this drug in an adult patient who received chemotherapy drugs: dasatinib, imatinib, and nilotinib, and anti-CD19 CAR-T cell therapy. Before blinatumomab application, the patient with Ph-positive R/R B-ALL had relapsed and had TKI-induced side effects after receiving these drugs. The combinational application of blinatumomab and ponatinib, another tyrosine kinase inhibitor, had paved the way for the complete remission for twelve months. However, the patient had developed a central nervous system relapse of ALL and pneumonia and died due to respiratory failure. This CNS relapse can be sourced by the off-target combinational activity of blinatumomab and T-cells (El Chaer et al., 2019). The patient history with this type of multieffector therapies should be examined day by day to obtain better results and an explanation of personalized cancer immunotherapy.

The enhanced success in personalized cancer immunotherapies for hematological malignancies and solid tumors will increase the overall survival median and life qualities of the patients in near future of biomedicine. The advanced genomic profiling studies of tumor cells and pharmacogenomics may shed light on the differences between cancer and noncancerous cells and drug activation and efficacies, thereby improving combinational CAR-T cell therapies for each carcinoma.

23.3.5.2 CAR-NK cells

Natural killer (NK) cells are a kind of lymphocyte, which have various similarities with cytotoxic T cells for adaptive immune response after infection and tumor formation. Therefore NK cells can be used as effector cells in cancer immunotherapies. In addition to CAR-T cells, CAR-NK cells have recently been used in personalized immunotherapies due to their significant advantages. CD19-targeting CAR-T cells can eliminate noncancerous CD19-bearing B cells when the lifespan of CAR-T cells is prolonged to increase the efficacy of immunotherapy. However, the lifespan of CAR-NK cells is limited in the blood circulation, thereby reducing their on-target/off tumor effects. Secondly, CAR-NK cells secrete different cytokines such as IFN-γ and granulocyte/macrophage colony stimulating factor (GM-CSF) for promoting immune response while CAR-T cells produce IL-1, IL-6, and TNF-α, thereby resulting in the cytokine release syndrome. Thirdly, CAR-NK cells are able to kill cancerous cells via CAR-specific and NK cell cytotoxicity receptor (CD16, CD226, NKp30, and NKG2D)-specific mechanisms (Hu, Tian, & Zhang, 2018), while cells in the tumor mass are evolving and changing their genomic profiles due to the CSC hypothesis or responses to treatments. However, CAR-T cells cannot eliminate this type of tumor mass because of their first-round effect on cancerous cells (Maude et al., 2014).

CAR-T cell immunotherapies have provided promising results on the precursor-B cell ALL, and furthermore, based on the advantages of NK cell lines, NK-92 cells have been recently used in B-ALL treatment modified with CAR against FMS-like tyrosine kinase 3 (FLT3 or CD135), which are overexpressed in B-ALL cells. FLT3-targeting CAR-NK cells specifically recognize the FLT3-positive B-cells and then release their cytotoxic molecules, named degranulation, through the B-ALL cells in vitro. The in vivo experiment of FLT3-targeting CAR-NK cells has demonstrated that CAR-NK cells have exhibited higher antitumor activity inhibiting the leukemic progression in a B-ALL xenograft NOD-SCID IL2R γ^{null} (NSG) mice model. Besides, these CD135-targeting CAR-NK cells are derived from NK-92 cells, which have tumor origin and aneuploidy. Thus they must be irradiated before their infusion into the host tissues. This research study has used a suicide gene, inducible caspase-9 (iCasp9) while transducing with a lentivirus transfer plasmid by combining CD135 and iCasp9 genes for a suicide switch mechanism to provide the safety of CAR-NK cell immunotherapy. The coexpression of both (iCasp9 is induced by inert chemicals) effectively eliminated the cancerous B-cells for better treatment (Oelsner et al., 2019).

In addition to tumor antigen-targeting studies about B cell malignancies, T cell acute lymphoblastic leukemia (T-ALL) studies are still challenging in clinical trials for antigen-targeting malignancies. CD7 is overexpressed in T-ALL and the research group designed CD7-targeting CAR-NK-92MI derived from NK-92 cells transfecting IL-2 gene. CAR-NK-92MI application to CD7-positive cell lines and primary T-ALL cells has documented the significant antitumor activity in vitro. Monoclonal mdCD7-CAR-NK-92MI cells can produce efficiently granzyme B and IFN-γ to elevate the cytotoxicity in T-ALL cells. Moreover, mdCD7-CAR-NK-92MI cells efficiently recognize and eliminate T-ALL cells in the xenograft mice, which are derived from patients and transplanted into the mice. This study has documented

for the first time that CD7 targeting CAR-NK-92MI cells inhibit the proliferation of patient-derived T-ALL cells in the xenograft mouse models (You et al., 2019). Besides CAR-NK cell studies in hematological malignancies, CAR-NK cells are recently used for the first time in glioblastoma as a solid tumor immunotherapy study. EGFRvIII-targeting CAR-KHYG-1 (a human CAR-NK cell line) is generated through lentiviral transduction of EGFRvIII gene named EvCAR-KHYG-1 cells and they are enriched using magnetic bead sorting method. This research group has documented that engineered cells inhibit cellular growth of EGFRvIII-positive glioblastoma cells by promoting apoptosis (Murakami et al., 2018).

The significant results in engineered NK-92 cells provide the potential usage of these engineered NK cells in personalized cancer immunotherapies due to their "off-the-shelf" production under sufficient conditions but their usage as engineered cells needs irradiation process because of their tumor cell origin (Hu et al., 2018). Conversely, NK cells derived from human embryonic stem cells (hESC) and human induced pluripotent stem cells (iPSCs) can be modified easily to produce CAR-expressing NK cells. Secondly, NK cells can be modified to enhance in vivo persistence and antitumor activity using the CRISPR/Cas9 system in addition to the lentivirus application (Wang, Han, Cho, & Zhu, 2018). A hiPSC-NK study using xenograft ovarian cancer model has documented that NKG2D transmembrane domain bearing CAR-expressing iPSC-NK cells downregulate the ovarian tumor growth and prolong the mouse survival. CAR4-iPSC-NK cells are significantly persistent in the blood circulation, spleen and peritoneal fluid comparing to peripheral blood (PB) NK and nonengineered iPSC-NK cells on day 10. Moreover, CAR-4-expressing iPSC-NK cells have shown the highly promising results inhibiting side effects on ovarian xenograft tumor mouse model, thereby CAR3-iPSC-NK received mice have prolonged survival with no obvious weight loss and damage on the organs (Li, Hermanson, Moriarity, & Kaufman, 2018). This approach can elevate the efficacy of personalized cancer immunotherapy for refractory malignancies by combining other treatment ways in clinics.

Challenges in clinical applications of CAR-Natural killer cells in tumor immunotherapy give rise to limit the efficacy of human trials. The major challenge is the ex vivo expansion and purification of antigen-targeting human CAR-NK cells. The safer way of overcoming this challenge includes their activation with cytokines and derivation from hESCs or iPSCs (Hu et al., 2018). Due to the irradiation of NK-92 cells, these cells show lower persistence in the body circulation, thereby resulting in limiting the antitumor activity of CAR-NK cells. The releasing of immunosuppressive cytokines or metabolites from tumor microenvironment give rise to dysfunction or exhaustion of human engineered NK cells. The presence of CAR-NK cell activation receptors is the major improvement in the overcoming challenges such as immunosuppression by TME. The enhanced activating receptor bearing NK cells can be generated by hESCs/iPSCs due to their convenience of genetically engineering using transposons, lentiviruses or CRISPR-Cas9 to induce antitumor activity for personalized cancer immunotherapy (Wang et al., 2018).

Although there are many improvements and challenges in CAR-NK cells, this area sheds lights on the treatment of personalized hematological and solid malignancies as an alternative therapy for future biomedicine. The developments in the intellection of CAR-NK cells in clinical studies will help patients to eliminate cancerous cells specifically after uncovering the genomic profiling of cancerous cells.

23.3.5.3 Macrophages for regulation of tumor microenvironment

Cancer immunotherapy has exhibited a significant progression using CAR-T and CAR-NK cells for the treatment of cancerous patients but their activities are restricted by immunosuppressive TME metabolites. Macrophages are a kind of white blood cells that contribute to innate immune system response as M1 and M2 classes. Tumor-associated macrophages (TAM) or M2 are involved in tumorigenesis and progression of cancerous cells due to their immunosuppressive functions. The deficiency of TAMs in TME or TAMs reprogramming may be an efficient strategy to enhance antitumor activity. MicroRNA 155-loaded lipid-coated mannose conjugated pH-responsive calcium phosphate nanoparticles can reprogram TAMs, thereby reversing immunosuppressive TME by directly inhibiting their target gene expression involved in TAMs activation and enhancing the inflammatory cytokine production. The highly expressed mannose receptors on TAMs are selective biomarkers for TAM reprogramming to antitumor macrophages. The mannose and its receptor interaction provide miR-155 secretion under suitable acidic TME conditions. Thus miR-155 can reverse TAMs phenotype and these macrophages produce IL-12 that suppresses tumorigenesis in S180 sarcoma cells-bearing mouse model. This study reveals that the reprogramming of TAM polarization using effective carriers may be a potential supportive therapeutic strategy for personalized cancer immunotherapy (Zang et al., 2019).

In addition to the redirecting of TAMs polarization in TME for alternative therapeutic strategy, the phagocytic activities of macrophages can also be reprogrammed in the TME to kill cancerous cells. Cancerous cells widely overexpress the CD47 surface marker to bind SIRPα, inhibitory receptor signal regulatory protein alpha on phagocytic cells for

mediating "don't eat me" signal through phagocytic cells. Indeed, CD47 can also bind to thrombospondin-1 (TSP1) protein on T cells, thereby resulting in suppression of T cell activation. The developed antibodies for CD47 blockade enhances the phagocytic activity of macrophages against antibody-bound cancer cells. CD47 targeting immunotherapy has demonstrated that plant virus nanoparticles (VNPs) generated by the cowpea mosaic virus (CPMV) as immune-stimulators promote phagocytic and antigen-presenting abilities of the innate immune cells for enhanced cancer immunity. VNPs as in situ vaccines were applied to CD47-expressing 4T1 breast and ID8-Defb29/Vegf-A ovarian cancer cells respectively inoculated BALB/c or C57BL/6 mice. Thus the cytotoxicity of macrophage has increased to kill tumor cells. The combinational therapy CD47-blockade antibody and VNPs resulted in enhanced efficiency to kill both cancerous cells via the efficient macrophage activation in vitro. This combinational therapy to breast cancer cells in vitro was proven in vivo mouse model whereas this combinational therapy to CD47 expressing ovarian cancer cells was not correlated with in vivo mouse model because of different TME and physiology of both cancer models. Collectively, this combinational study reveals for the first time the usage of macrophage activity and VNPs as immune-stimulators of innate immune system (Wang & Steinmetz, 2019). Therapeutic strategies parallel with adoptive T-cell therapies or immune checkpoint inhibitors may enhance the survival and life qualities of cancer patients as main topics of current age biomedicine.

Adoptive immunotherapies have exhibited promising results for the treatment of cancer patients. However, their efficacies in TME are restricted by immunosuppressive cytokines released by TAMs. Cancerous cells release colony-stimulating factor 1 (CSF-1) and IL-10 through TME to recruit naive macrophages and transform them to TAMs. This CSF-1 cytokine interacts with its receptor Colony-stimulating factor 1 receptor (CSF1R) and modulates the conversion of naïve macrophages to M2 TAMs that significantly secrete IL-10 and TGF-β to inhibit the immune regulation in TME. Therefore cancerous cells recruit macrophages to support themselves secreting these cytokines to suppress contemporary adoptive immune therapies. A research study revealed that CSF1R-expressing K562 CML cells are specifically killed by CSF1R-targeting CAR-NK92MI and CAR-T cells in vitro. A coculture experiment of CAR-T, CAR-NK, and CSF1R-expressing K562 cells revealed that these third generation CAR-T and CAR-NK cells significantly secreted IFN-γ and granzyme B to kill engineered K562 cells. Furthermore, these CAR-T and CAR-NK cells have no toxicity on monocytes derived from human peripheral blood and have expressed CSF1R. The reason for lower toxicity on these monocytes might be their low average expression in peripheral blood (Zhang, Zhao, et al., 2018). These monocytes might not possibly be activated by tumor cells and their CSF1R expression levels are not to recognized and killed by these engineered CAR-T and CAR-NK cells in vitro. The CSF1R-targeting CAR-T and CAR-NK cells could demonstrate novel alternative personalized immunotherapy strategies to eliminate the TAMs in TME, thereby resulting in the inhibition of tumor growth, cell proliferation, immunosuppressive cytokine secretion, and metastasis. The combination of this type of therapy with distinct eradication therapies for cancerous cells in TME may be a promising solution in near future biomedicine.

Another combination approach includes merging activities of the oncolytic virus and CAR-T cells in pancreatic ductal adenocarcinoma (PDA). Mesothelin (meso) is overexpressed in pancreatic cancers and can be a target for CAR-T cells. The oncolytic adenoviruses can highly express TNF-α and IL-2 for efficient immune system response. The combinational therapy of mesotargeted CAR-T cells with these oncolytic viruses may increase secretion of these cytokines from infected tumor cells, thereby enhancing the M1 macrophage polarization. Thus enhanced CAR-T cell activity through TME can inhibit progression of immunosuppressive and aggressive PDA tumors. This type of combinational studies may be a promising strategy to suppress the immunosuppressive TME of PDA tumors (Watanabe et al., 2018).

Although contemporary adoptive immune therapies have prolonged patient survival and life qualities, there are still challenging problems such as immunosuppressive cytokines secreted by tumor cells and TAMs and immune checkpoints. The adaptation of macrophage-based alternative strategies with these clinically proven adoptive therapies may enhance the eradication of hematological and solid malignancies in the clinical studies for personalized cancer medicine.

23.4 Future perspectives and conclusion

Cancer cells are able to divide continuously without any control, rendering the immortal cells with many changes in their DNA sequences, thereby resulting in dynamic instability. Therefore they have altered their marker expression; marker clusters, cellular signaling pathways, cellular division, and cellular growth-related genes or proteins, and new synthesis of tumor-specific transcripts such as mRNAs and microRNAs. Indeed, their response to the cellular microenvironment can cause temporary or permanently epigenetic changes in their genomes, resulting in carcinogenesis.

These changes also aid cancerous cells to evade tumor suppressor proteins and immune system cells. Collectively, each cancer cell in a tumor mass actually has various changes for the specific tumor type.

Cancer is one of the major causes of death on the Earth and 1 in 10 women and 1 in 8 men are going to die because of cancer. The GLOBOCAN 2018 database revealed that there were 18.1 million people diagnosed as cancer patients and 9.6 million patients die because of cancer worldwide each year. Nearly 1.8 million cancer patients worldwide will die because of lung cancer. Similarly, the estimated number of new cases of female breast cancer patients is approximately 2.1 million and about 600,000 women will die from breast cancer in the year 2019.

Current treatment strategies for the novel cancer cases have had limited success in removing cancerous cells in patients. Personalized therapies in biomedicine seek differences in cancerous cells compared to the adjacent normal cells, as discussed above in detail. However, cancerous cells have the ability to alter different molecules to proliferate continuously and to escape from suppressor molecules or cells. The detection, progression, and influences of these differences on cancerous cells are getting easier but the foe for our lives are becoming stronger by negatively regulating treatment agents in their microenvironments. Within this context, the most effective treatment methods include combinational teamwork of current or novel treatment strategies to screen, prevent, and abolish cancer cells. Genetic profiling of cancerous cells compared with adjacent normal cells using sequencing methods of whole genome and exome and tumor-related RNAs, genes, and proteins could demonstrate the root cause of developing cancer in a patient. Therefore the progression of the tumor may be visualized; the best treatment option without any side effects may be carried out to eliminate the recurrence risk of cancer. After determination of alterations in cancer cells using genomic profiling techniques, the efficient personalized medicine strategies should determine the drug efficiency in the cellular metabolism before/during/after application using bioinformatic tools. Tumor-specific changes in the body circulation such as tumor-causing genes and stem cells, circulating tumor cells, and immune suppression should be determined to keep up with the alterations in cancerous cells of tumor mass and to modify the treatment strategies. The life of a patient or its quality depends on a clinician's opinion and the decision as to whether she/he can realize these alterations for the treatment. The treatment for a Ph-positive ALL patient can start with chemotherapy agents and continue with CAR-T cells and achieve at the end a three member-combinational study—TAMs-targeting CAR-NK cells, tumor cell-targeting CAR-T cells, and CD47 blocking agent—in harmony to save patient lives. However, saving lives needs passion, determination, and the tears of nobody who lives in a laboratory.

Collectively, cancerous cells have an ability to grow without any regular control and to evade many regulators as well as circulate in the blood. There are many developed strategies to eradicate cancerous cells naturally or artificially. However, cancerous cells have highly dominant features, whose progressions are hard to follow. Since artificial intelligence is predominantly in our lives, the machine learning of this intelligence can help researchers and clinicians based on the data come from various laboratories, numerous assays, and treatment methods. Therefore data about cancerous cells compared with noncancerous cells obtained from combinational studies which target the cancerous cells from different windows will help to treat cancer patients.

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Chapter 24

Vascular and bone marrow explant models to assess in vitro hematotoxicity of herbal extracts

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24.1 Background

Mankind has exploited the therapeutic potential of plants for mitigating human diseases since antiquity (Neergheen-Bhujun et al., 2017). The earliest records of medicinal plants date back to the Sumerian clay slabs about five millennia ago (Hassan, 2015). At least 28,000 plant taxa worldwide are currently documented as medicinal (State of the World's Plants, 2017). Even in this modern era of a well-established mainstream medicine, about 80% of the developing world population continues to rely on the plant kingdom for primary care in alleviating human ailments (Chen et al., 2016). The predominant dependence upon medicinal plants in developing countries is fueled by the lenient marketing regulation of herbal remedies (State of the World's Plants, 2017; Stournaras, 2015). As such medicinal plants are more easily accessible and affordable to the general population compared with conventional drugs. However, the practice of herbalism is based on empirical findings, within the majority of cases no proven therapeutic and toxicity profile (Gurib-Fakim, 2006). Although herbal remedies are generally perceived to be free from potential adverse effects, (Fatima & Nayeem, 2016; Neergheen-Bhujun, 2013; Stournaras, 2015; Zhang, Onakpoya, Posadzki, & Eddouks, 2015), the fatalities emanating from their consumption cannot be underestimated. Growing evidence reports their severe toxicity to different organ systems (Stournaras, 2015; Teschke, Frenzel, Glass, Schulze, & Eickhoff, 2013). Exposure to herbal, including dietary and homeopathic, supplements were identified among the major causes of poisoning in the United States (Mowry, Spyker, Brooks, McMillan, & Schauben, 2014). A case report from Réunion Island shed light on intoxication resulting from a refreshing herbal decoction, leading to severe hypotensive effect (Lopez, Drouet, & de Haro, 2013). Similarly, hospitalization of pediatric patients following poisoning from *Jatropha curcus* (Euphorbiaceae), a commonly used medicinal plant in Mauritius, has been reported (Rai & Lakhanpal, 2007). Therefore evaluating the safety and toxicity window of herbal extracts remains crucial to establish their risk:benefit ratio for human consumption.

Herbal remedies remain an integral part of the complementary and alternative medicine in Mauritius, as evidenced by numerous ethnopharmacological surveys (Sreekeesoon & Mahomoodally, 2014; Chintamunnee & Mahomoodally, 2012; Mahomoodally & Muthoorah, 2014; Mootoosamy & Mahomoodally, 2014; Nunkoo & Mahomoodally, 2012). Medicinal plants are documented to alleviate pathologies ranging from diabetes mellitus, hypertension, skin diseases, and arthritis to sexually transmissible diseases (Gurib-Fakim & Brendler, 2004; Gurib-Fakim, Gué, & Bissoond, 1995, 1996, 1997; Rouillard & Guého, 1999; Sreekeesoon & Mahomoodally, 2014). The use of native plants, including

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endemic taxa, likely paralleled human colonization of the island since 1638 (Cheke & Hume, 2008; Rouillard & Guého, 1999). Leaves of the Mauritian endemic *Eugenia tinifolia* Lam (Myrtaceae) and *Acalypha integrifolia* Willd (Euphorbiaceae) are applied topically to treat a myriad of dermatological conditions (Gurib-Fakim & Brendler, 2004; Gurib-Fakim et al., 1996). Furthermore, the leaf extract of the latter is also ingested orally for its anthelmintic activity (Mahomoodally & Aumeeruddy, 2017; Seebaluck, Gurib-Fakim, & Mahomoodally, 2015). The bark extract prepared from *Labourdonnaisia glauca* Bojer (Sapotaceae) is administered orally for its antihemorrhagic effects (Gurib-Fakim & Brendler, 2004; Gurib-Fakim et al., 1997). Even though a number of studies have reported the in vitro bioactivities of selected endemic medicinal plants (Aumeeruddy-Elalfi, Gurib-Fakim, & Mahomoodally, 2015; Neergheen, Bahorun, & Aruoma, 2011; Neergheen, Soobrattee, Bahorun, & Aruoma, 2006), limited insight about their safety and toxicity profile for human consumption is available in the literature to date, which remains a subject of major concern.

The hematopoietic cells comprise one of the most proliferating compartments of an animal's body and are vulnerable to damage following ingestion of exogenous toxicant; these cells can be used as a sensitive in vitro model for the screening of plant extracts with a view to predicting their in vivo toxicity profile (Clarke et al., 2007). Since ex vivo culture of hematopoietic cells can be maintained in the absence of growth factors using organ explants and coaggregates with the stromal cells line, we used such a model to test medicinal extracts' influence on the hematopoietic toxicity, proliferation and blood lineages differentiation (Forcados, Chinyere, & Shu, 2016; Sheridan, Taoudi, Medvinsky, & Blackburn, 2009). Taking advantage of previously developed efficient hematopoietic ex vivo culture and culture of day 11 mouse embryonic aorta and cKit + bone marrow progenitors coaggregated with OP9 osteoblastic supportive cell line, we examined the influence of plant extracts on the adult and embryonic hematopoietic and endothelial cells. We suggested this approach as a useful method for the preliminary toxicity analysis of herbal extracts prior to designing an in vivo experiment using live animals.

24.2 Material and methods

24.2.1 Plant extracts preparation

Fresh leaf samples of the plants (Fig. 24.1) were collected and a voucher specimen was deposited at the local herbarium. The leaves of *L. glauca* and *A. integrifolia* were collected at Gaulette serré, near Camp Thorel (coordinates 20° 12′ 09″ S, 57° 25′ 11″ E and 20° 12′ 43″ S, 57° 38′ 29″ E, respectively), while the leaves of *E. tinifolia* were collected at lower gorges national park, "Morne Sec" (coordinates 20° 23′ 35″ S, 57° 38′ 05″ E). Then dried leaves were extracted as described by Rummun, Somanah, Ramsaha, Bahorun, and Neergheen-Bhujun (2013). Finally, lyophilized extracts were dissolved in DMSO for subsequent analyses.

The extracts were further diluted and tested at the concentrations of *A. integrifolia* 25 μg/mL (A25) and 5 μg/mL (A5); *E. tinifolia* 25 μg/mL (E25) and 5 μg/mL (E5); *L. glauca* 25 μg/mL (L25) and 5 μg/mL (L5) (Figs. 24.3 and 24.4). Media was supplemented with the extract, before culture started, in an appropriate concentration expressed in μg of lyophilized dry weight per mL of the media. The explants were cultured for 5 days (Fig. 24.2). Control media was supplemented with the same concentration of DMSO.

24.2.1.1 Hematopoietic tissues and cells isolation

E11 embryos were obtained by crossing C57BL/6 mice. On day 11 postfertilisation pregnant dams were sacrificed and the embryo was isolated. Following 3R policy, bone marrow was isolated using the same animals. Embryonic dorsal aorta region was isolated using dissection needles and a LEICA binocular microscope as described (Bertrand, Giroux, Cumano, & Godin, 2005; Rybtsov et al., 2014).

24.2.1.2 cKit + cell isolation

Bone marrow cells were flashed out from B6 mice bones, pipetted, and filtered prior to the cKit + cells isolation by antimouse CD117 Micro Beads. Beads application and magnetic separation with LS columns were done in accordance with the manufacturer's protocol (Myltenyi Biotec, United Kingdom).

24.2.1.3 In vitro cell culture

Two cell models were used to test the influence of the extracts on cell development and proliferation: (1) embryonic dorsal aorta region explants known to produce large numbers of the hematopoietic stem/progenitor cells with highly proliferative and differentiation potential; and (2) cKit + cells from adult mouse bone marrow, highly enriched in the

FIGURE 24.1 Endemic medicinal plants used in this study. The herbarium voucher barcode number is indicated in brackets.

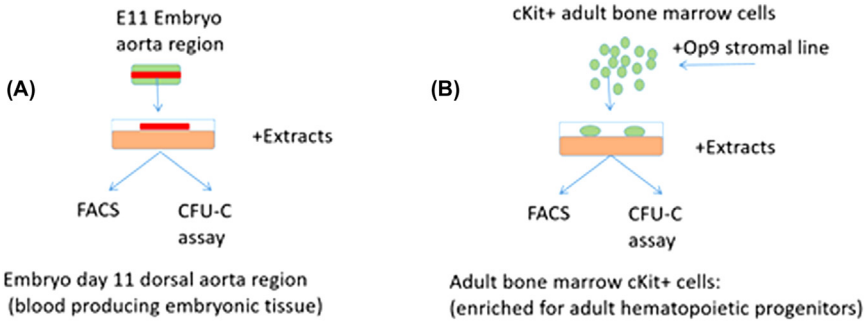

FIGURE 24.2 Outline of the study. (A). Day 11 embryonic dorsal aorta explant assay (B). Adult cKit + bone marrow cell coaggregates with op9 stromal cell line. After 5 days of culture in presence of extracts cells were isolated by collagenase/dispase treatment. Differentiation and number of cells were tested by FACS and by methylcellulose assay for estimation the number of the myeloid progenitors (CFU-Cs).

adult hematopoietic stem/progenitor cells. Isolated tissues and cells were cultured in the liquid−air interface using previously established conditions: nitrocellulose membrane and Iscove's modified Dubelco's medium (IMDM) supplemented with 20% fetal calf serum, glutamine, penicillin/streptomycin, and extracts as described above. We coaggregated 50,000 adult bone marrow cKit + cells with 100,000 OP9 stromal cells, a total of five coaggregates per variant (250,000 cKit + cells combined). A detailed culture protocol was described previously (Rybtsov et al., 2011; Taoudi & Medvinsky, 2007). E11 aorta explants were cultured in the same media as established previously (Sheridan et al., 2009) (Fig. 24.2). After 5 days of culture in the presence of extract or control media, cells were dissociated by collagenase/dispase treatment, as described earlier (Nakano, Kodama, & Honjo, 1994), and processed by fluorescence-activated cell sorting (FACS) analysis to estimate the number of cells in each of the distinct hematopoietic cell populations.

24.2.1.4 *Colony-forming unit cells*

The number of colony-forming unit cells (CFU-C) was evaluated after culturing in methylcellulose medium for embryo aorta explants (Fig. 24.3E) and adult cKit + cells (Fig. 24.4E). This assay is used to establish the numbers of the clonogenic myeloid progenitors, which we consider to provide clinically relevant information on the potential toxic effects of the novel compounds (Clarke et al., 2007). For this assay, an aliquot of the cell suspension was seeded onto the 30 mm untreated plastic dishes and grown within the methylcellulose medium (M3434; Stem Cell Technologies) following the manufacturer's protocol. Hematopoietic colonies were counted according to their morphology as mixed colonies

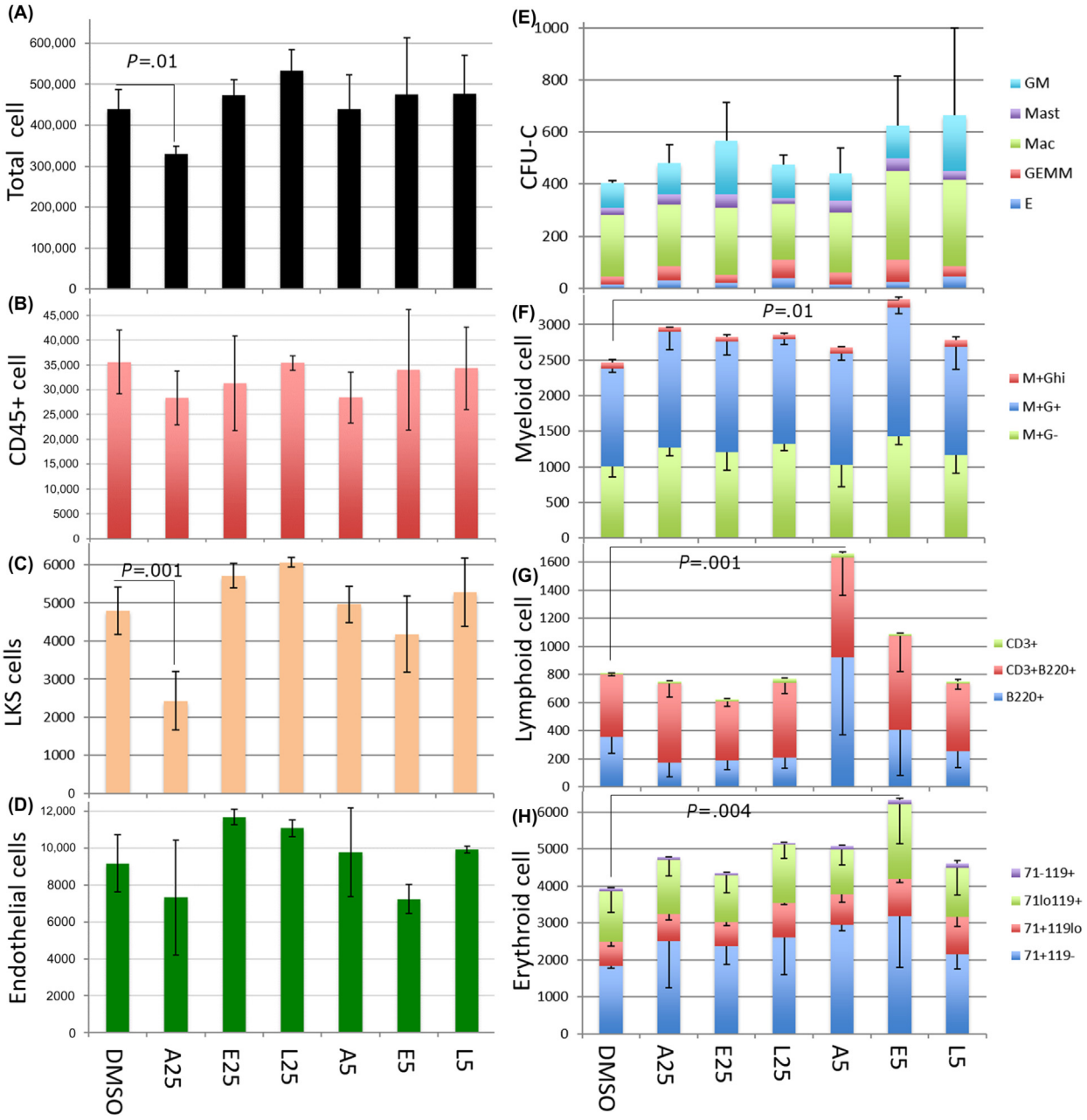

FIGURE 24.3 Effects of plant extracts on hematopoietic cell and endothelial development in the E11 embryonic aorta culture. Total cell number (A). The number of hematopoietic (CD45 +) cells (B). The number of hematopoietic stem/progenitor cells LKS (lineage negative, cKit + Sca1 +) (C). The number of endothelial cells (VE-Cadherin + CD45-lineage-) (D). The number of myeloid progenitors (CFU-C). (E) The number of differentiated myeloid cells. Mac1, Gr1 (F). The number of differentiated lymphoid cells CD3, B220 (G). Number of cells from erythroid lineage CD71, Ter119 (H). All figures represent number of cells after 5 days in vitro per one embryonic aorta. (Three independent experiments).

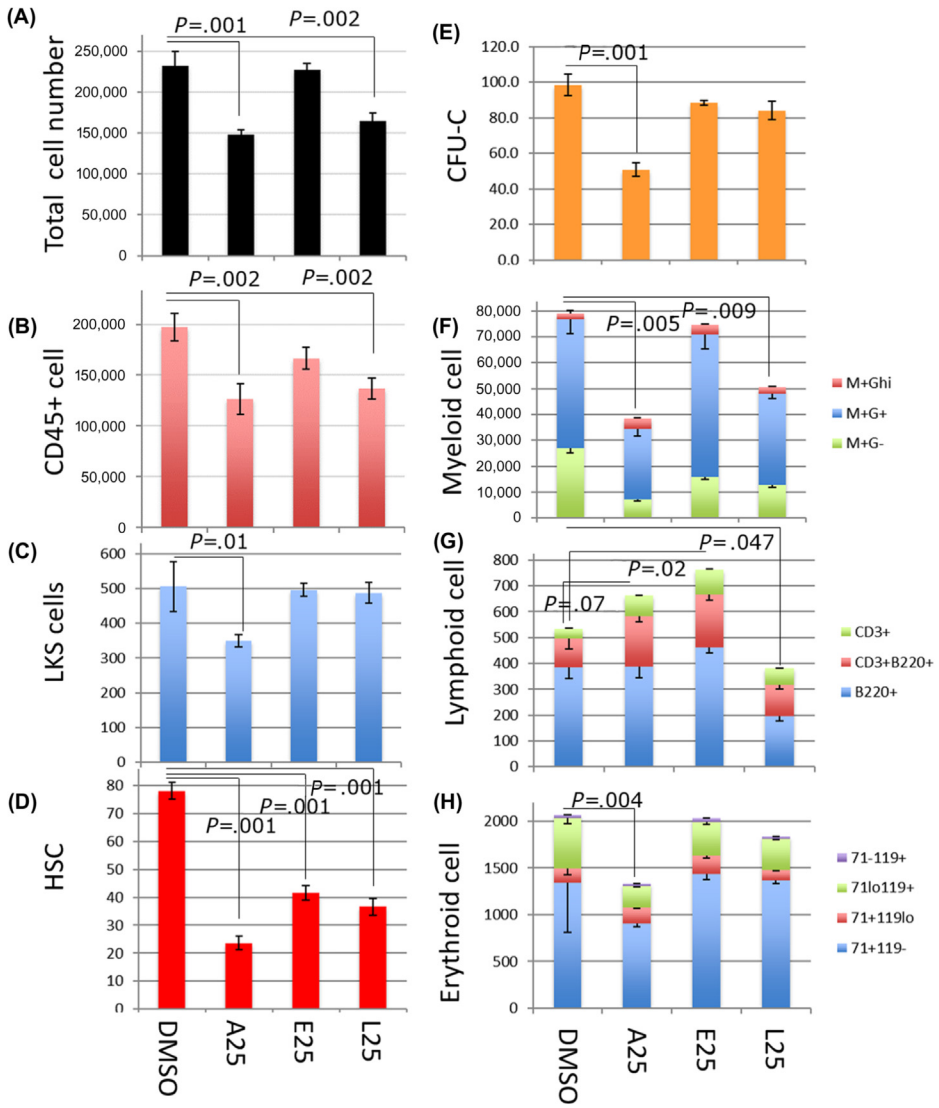

FIGURE 24.4 Effects of plant extracts on bone marrow-derived adult progenitor proliferation and differentiation *ex vivo*. Total cell number (A). Hematopoietic (CD45 +) cell number (B). Hematopoietic stem/progenitor LKS cells (C). Hematopoietic stem cells number (Lin-LSK CD48-CD150 +) (D). The number of myeloid progenitors (CFU-C) (E). The number of differentiated myeloid cells Mac1, Gr1 positive (F). The number of differentiated lymphoid cells CD3, B220 positive (G). The number of cells from erythroid lineage CD71, Ter119 positive (H). All figures present number of cells after 5 days in vitro. Number of cells after culture given for 250,000 of cKit + input (A–D, F–H) and 10,000 cKit + cells input for (E). All data representative of three independent experiments.

containing granulocytes and macrophages (GM colonies), macrophage colonies (M), mast cell (Mast), multipotent granulocytes–erythrocytes–macrophage–megakaryocytes colonies (GEMM), and erythroid colonies. Colonies were scored twice on days 7 and 11 of the culture.

24.2.1.5 Fluorescence-activated cell sorting analysis

After culture cells were isolated using collagenase/dispase as described (Nakano et al., 1994), cells were stained with the cocktail of antibodies: For myeloid (Mac1/CD11b, Gr1), lymphoid (CD3, B220), and erythroid (Ter119, CD71) with the exclusion of CD11b, Gr1, CD3, B220 positive cells (all from BD Bioscience). Dead cells were excluded by the 7AAD staining (Invitrogen). FACS analysis was performed using a BD LSR-Fortessa instrument. Data were analyzed using FlowJo software (FlowJo LLC).

24.3 Results and discussion

The aim of this study was to test the effects of the extracts prepared from the selected endemic medicinal plants of Mauritius on hematopoiesis and proliferation of the endothelial cells in vitro, with a view to predict their hematotoxicity and safety profile for in vivo studies. Extracts from *A. integrifolia*, *E. tinifolia*, and *L. glauca* were tested on embryonic cells and adult bone marrow cells at two different concentrations of 5 and 25 μg/mL. We have explored the influence of

the plant extracts on the cells of the embryonic dorsal aorta isolated from E11 mouse embryo and grown in the liquid−air interface explant culture. In addition, the effects of the same extracts were studied on adult bone marrow cKit + hematopoietic progenitors (Fig. 24.2). Isolated cKit + progenitors were coaggregated with the osteocyte-like stromal cell line OP9, which provides the minimal niche for the hematopoietic progenitor's survival and differentiation (Taoudi & Medvinsky, 2007; Taoudi et al., 2008). cKit ligand (SCF), IL3, and Flt3L were used in control experiments as strong hematopoiesis agonists (Wang et al., 2013) to demonstrate the sensitivity of the in vitro testing system applied in this study. We scored the changes in the selected cellular markers which allowed us to establish the distribution of the principal blood cell components and to estimate any changes in their numbers after 5 days culture (Figs. 24.3 and 24.4). Gating strategy for embryonic and adult cells after culture is shown in supplementary material.

No significant changes in the embryonic aorta culture in total cell number and in the number of the CD45 + hematopoietic cells were observed following the treatment with either of the extracts in this study. However, a slight reduction in the total cell number upon treatment with *A. integrifolia* extract was indicated ($P = .01$) (Fig. 24.3A and B). The adult progenitors have shown a more pronounced decrease in their number upon treatment with either *A. integrifolia* or *L. glauca* extract at 25 μg/mL (Fig. 24.4A and B). This effect can be explained by the significantly more powerful proliferative ability of the embryonic cells in comparison with the adult cells. Moreover, the progenitor/stem population (LKS) was significantly reduced after *A. integrifolia* treatment in both experimental systems tested (Figs. 24.3C and 24.4C). No significant drop in the cell number was observed for the total adult cKit + from bone marrow after culturing them in the presence of the *E. tinifolia* extract compared to the control. Incubation with *A. integrifolia* and *L. glauca* extracts at the highest experimental concentrations led to 20%−30% reduction in the numbers of the total adult cKit + bone marrow cells ($P = .001$ and $P = .002$, respectively) and differentiated CD45 + cells ($P = .002$) (Fig. 24.4A and B). Moreover, we further tested the number of HSC (cKit + Sca1 + CD48- CD150 + Lin-) in adult progenitor's model where the incubation with each of these three extracts significantly decreased this essential population (Fig. 24.4D). No significant changes in the endothelial cells number were observed in our study (Fig. 24.3D).

The influence of the extracts under study on the stem cells differentiation into the principal blood lineages was analyzed by separately estimating the number for each of the myeloid, lymphoid, and erythroid cell populations.

Unexpectedly, even at a low concentration of 5 μg/mL, *A. integrifolia* stimulated the development of the lymphoid cell lineage, while *E. tinifolia* stimulated the development of both erythroid and myeloid cells. Considering the possible toxic effects of the *A. integrifolia* extract on the total and CD45 + cells in high concentration (Fig. 24.3A and C), the increase in the lymphoid and myeloid cells only upon treatment with lower doses, led to the suggestion that small concentrations of the constituents of *A. integrifolia* and *E. tinifolia* extracts could be considered for future studies aimed at stimulation of the hematopoiesis. This "Janus" effect could be explained by the superposition of the distinct phytoconstituents present in the extracts, whereby at low concentration stimulatory effect was dominant, while toxic effects were taking over at higher doses. The "Janus" nature of herbal medicine is reported and provides potential avenues for treatment strategies against immunocompromised diseases (Mersch-Sundermann et al., 2006). Along a similar line, Mersch-Sundermann and coworkers reported the "Janus" potential of the Mauritian endemic *Monimiastrum globosum* (Myrtaceae), to protect hepatocellular carcinoma (HepG2) cells against genotoxicants at low doses, while inducing genotoxicity at elevated concentration (Ehiaghe, Ehiaghe, Bright, & Roland, 2013). At the higher concentration of each extract, inhibition of differentiated B cells (B220 +) production was observed. At the same time at lower concentrations, these extracts stimulated the production of B cells and their progenitors (Fig. 24.3G).

All extracts showed the tendency to increase the number of differentiated myeloid cells and myeloid progenitors in embryonic culture. *E. tinifolia* has a stronger tendency to promoting multipotent myeloid−erythroid progenitor proliferation at the lower concentration tested (CFU-GEMM, Fig. 24.3E); it is further confirmed by the FACS analysis estimation where we observed an increase in the number of the CD71lo Ter119 + differentiated erythroid cells (Fig. 24.3H) and myeloid cells (Fig. 24.3F).

In the adult model (Fig. 24.4) the reduction of myeloid cell number was observed at high concentration of *A. integrifolia* and *L. glauca* extracts treatment (Fig. 24.4F). However, the reduction in hematopoietic stem cells progenitor population (LKS), erythroid cells, and the numbers of the clonogenic precursors (CFU-C) were indicated only upon the *Acalypha* sp. treatment (Fig. 24.4C, E, and H). *L. glauca* extract treatment led to 50% reduction in the number of the differentiated B cells (B220 +). On the contrary, *Acalypha* and *Eugenia* extract increased the CD3 + B220 + population (Fig. 24.4G), which suggests selective effects of these extracts on the adult lymphoid differentiation. In all cases, we observed a tendency to increase the number of CD3 + T-cells.

Hematopoietic stem cells form all types of blood cells including all the essential components of the immune system. Therefore any effects on their viability and proliferation in the adult, as well as in the embryo, are critical for the organism's survival and development. Attenuation of the blood cells' function could result in immunodeficiency, anemia,

and other pathologies. On the other hand, the ability to modulate the immune cells could be exploited to compensate for pathological defects. Moreover, hematopoietic cells are highly proliferative and therefore they are very sensitive to toxic substances, especially molecules affecting cell division.

Acalypha species were traditionally used for medicinal purposes in different parts of the world and have been excessively investigated for their toxicity (Forcados et al., 2016). *Acalypha wilkesiana* showed effects related to hematopoiesis. A significant increase in lymphocytes proliferation, decreased the amount of IgG and hemoglobin level after oral administration was observed in adult rats Ehiaghe, Ehiaghe, Bright, Roland (2013). In addition, Eugenia species in particular Eugenia jambolana has been reported for its antiinflammatory effects Donepudi, Aleksunes, Driscoll, Seeram, & Slitt (2012).

Given that the use of these plants does not follow any stringent established dose, the findings are valuable in particular on the mild decrease in hematopoietic cells, which may be intrinsically linked to some adverse events, related to these medicinal plants administration.

Furthermore, we did not observe any significant toxic effects of the studied extracts on the embryonic hematopoietic cells, however, some stimulation of myeloid−erythroid cells and T-cells was observed at a lower dose. The stimulatory effects of *A. integrifolia* and *E. tinifolia* extract on the B cells and the myeloid−erythroid cells nevertheless need further investigation. The stimulatory effects in the lymphoid cells compartment of the adult bone marrow progenitors warrant additional studies to determine the mechanisms underlying the observed effects as well as further efforts aimed at identifying the active molecules within the extracts responsible for the activity are needed. Besides that, it is the first time to our knowledge, that the effects of these endemic plant species extracts on the hematopoietic cells ex vivo have been described. Moreover, we have validated the feasibility studies for the cell phenotype-based screening of the natural compounds with respect to their effects on hematopoiesis.

24.4 Conclusion

Overall, the findings of the present study suggest that at low concentration (5 μg/mL) the leaf extracts of all three investigated endemic plants did not produce any significant toxicity in both models tested. Furthermore, these extracts display myeloid−erythroid and lymphoid cell stimulation and can be considered for further evaluation in animal models.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All experiments using ex vivo animal cells were performed under a project Licence granted by the Home Office (United Kingdom), approved by the University of Edinburgh Ethical Review Committee, and conducted in accordance with local guidelines.

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Chapter 25

Nature-inspired synthetic analogues of quorum sensing signaling molecules as novel therapeutics against *Pseudomonas aeruginosa* infections

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25.1 Introduction

The discovery of antibiotics, which revolutionized medicine in many ways, was a turning point in human history and undoubtedly is one of the most important events of modern times. Initially antibiotics were so successful fighting against many common bacterial diseases that they were thought to be eradicated for good (Penesyan, Gillings, & Paulsen, 2015). However, shortly after the first antibiotics were introduces into medical practice, development of specific resistance mechanisms in bacteria casted doubt on their therapeutic use. The same fate has eventually met nearly all antibiotics that have been developed and introduced into clinical practice—the use of "miracle drugs," as antibiotics were dubbed, has been accompanied by the rapid appearance of resistant bacterial strains that compromise them as therapeutics (Ventola, 2015).

Today, almost a century after the first patients were treated, the number of bacteria resistant to the antibiotics inevitably increases, thus making again bacterial infections a substantial threat (Davies & Davies, 2010; Ventola, 2015). According to predictions by 2050, due to the spread of the resistance, humanity could reenter a preantibiotic era (O'Neill, 2014). This crisis is of global proportions; therefore, the World Health Organization has classified resistance to antimicrobials as one of the 10 major threats to public health, alongside climate change and air pollution (World Health Organisation, 2019), and called for immediate actions. Antibiotics are not exclusively utilized in medical practice; actually, significant amounts are used in farms and agriculture to cure or even prevent infectious diseases, as well as to promote animal and crop growth (Higuera-Llantén et al., 2018), hence making the problem of antibiotics resistance even more alarming.

Of special concern are human and animal bacterial pathogens that have evolved into multidrug-resistant (MDR) forms following the antibiotics usage (Davies & Davies, 2010). Moreover, new opportunistic pathogens with low susceptibility to antibiotics are emerging outside the hospital settings, indicating that nonclinical environments are also sources of antibiotic-resistant bacteria (Martinez et al., 2008; Munita & Arias, 2016).

Recent data showed that antibiotic resistance genes have been circulating within the microbial pangenome for millennia (D'Costa et al., 2011), implying that the resistance is a natural phenomenon that precedes selective pressure imposed by clinical use of antibiotics as well as that these genes have natural roles other than conferring antibiotic resistance (Penesyan et al., 2015).

In contrast, the appearance of antibiotic-resistant bacteria and their wide spread distribution are the results of underuse, overuse, and misuse of these drugs, and as such, are not natural processes, but a man-made condition imposed on the environment (Davies & Davies, 2010).

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Historically, the search for antibacterial drugs was focused on free-floating bacteria, and thus resulted in the development of antibiotics that target individual bacterial cells (Penesyan et al., 2015). Accordingly, most of the studies on antibiotic resistance mechanisms have been focused on resistance at the cellular level, which can be either intrinsic (owing to the mechanisms present in all the strains of a given bacterial species) or acquired (Blair, Webber, Baylay, Ogbolu, & Piddock, 2014; Martinez et al., 2008). But, when discussing the antibiotic resistance phenomenon it is usually thought of as the expression of acquired resistance in bacteria that have initially been susceptible to the antibacterial agent(s) (Munita & Arias, 2016).

The cellular resistance develops as a result of mutations in gene(s) often associated with the mechanism of action of the compound and/or through horizontal gene transfer of resistance determinant(s) from other microorganisms. Generally, mutations leading to antibacterial resistance modify the antibiotic action via (1) modifications of the antimicrobial compound, (2) prevention of the compound reaching the antibiotic target, and (3) changes to and/or bypassing of target sites. Thus resistance emerging due to acquired mechanisms is diverse and differs in complexity (Blair et al., 2014; Munita & Arias, 2016; Penesyan et al., 2015). However, it has long been recognized that other mechanisms can help bacteria survive exposure to antibiotics. Nongrowing and slowly growing bacteria can escape from bactericidal effect of antibiotics that require active growth for killing. This trait, called tolerance, is the ability to survive under treatment without developing resistance (Levin-Reisman et al., 2017).

Although bacteria are usually thought of as free-living unicellular organisms, today it is considered that biofilm is the predominant lifestyle for microorganisms in nature. Biofilm is a multicellular community immerged in self-produced polymeric matrices, which can be attached to biotic or abiotic surfaces. According to the National Institutes of Health of the United States, more than 75% of infections in humans are caused by the formation and persistence of biofilms (Miquel, Lagrafeuille, Souweine, & Forestier, 2016). Bacterial biofilms are linked to a broad range of infections, from those associated with in-dwelling devices (catheters or prosthetic joints) to chronic tissue infections such as those occurring in the lungs of cystic fibrosis patients or chronic wounds.

One of the crucial features that distinguishes cells in biofilms from planktonic ones is their significantly lower susceptibility to antibiotic agents (Hall & Mah, 2017). In biofilms bacterial cells can be 10−1000 times less susceptible to the antibacterial agents compared with their free-living forms (Balcázar, Subirats, & Borrego, 2015). Antibiotic resistance and tolerance specific for biofilm are multifactorial and depend on the bacterial species and strain, growth conditions, the stage of the biofilm development, and the specific antibacterial agent (Hall & Mah, 2017). Unlike the mechanisms that confer antibiotic resistance and tolerance on the level of the individual cell that are well understood, there are still many unanswered questions related to the mechanisms of biofilm recalcitrance. Some of mechanisms responsible for biofilm resistance against antibacterial agents that most bacterial species share include antibiotic efflux pumps, molecules sequestering antibiotics, and matrix β-lactamases. On the other hand, biofilm tolerance is a result of reduced growth rate, presence of persister cells, and the mechanisms dealing with antibiotic-induced oxidative stress (Hall & Mah, 2017; Olsen, 2015).

Taken all together, it is obvious that the development of antibiotic-resistant pathogens has increased and led to the spread of life-threatening infections that cannot be fought with the existing repertoire of antibiotic agents. Reduction of improper and needless usage of antibiotics will help to slow down this process, but certainly it cannot stop it. Thus the introduction of novel therapeutic options to fight bacterial infections has become imperative. Despite the growing need for novel and more efficient antibiotics, development of new classes of such therapeutics has lagged far behind our expectations since antibiotic development has not been considered an economically wise investment for the pharmaceutical industry. In the last 30 years not a single new class of antibiotics has been discovered and every currently available antibiotic is a derivative of an older generation of antibiotics acting on the familiar, evolvable targets (Silver, 2011).

The current strategy often used by clinicians to treat MDR infections is utilization of different combinations of antibiotics and a lot of research has been focused on developing ideal antibiotic combinations to improve the efficiency of these treatments (Rahal, 2006; Sobieszczyk et al., 2004; Waters & Smyth, 2015). Additionally, efforts have been made to identify pharmacologically active compounds called adjuvants or potentiators that could be combined with antibiotics to increase or restore their activity. Opposite to antibiotic combinations, their adjuvants show little or no antibacterial activity alone. Adjuvants could be classified into two general classes: (1) adjuvants acting on bacterial targets together with antibiotics, and (2) the ones enhancing activity of antibiotic in the host (Wright, 2016).

Therapeutics that affect vital microbial functions, like conventional antibiotics, induce high selective pressure, and thus cause rapid occurrence of the resistance. One of the promising alternatives to the classic antibiotic practice, known as antivirulence therapy, has recently been established. Rather than targeting bacterial growth or viability, this alternative strategy targets bacterial virulence machinery required for host damage and disease. Bacteria are equipped with an arsenal of virulence factors, diverse in structure, function, localization, and regulation, and with secretion systems that

translocate virulence factors into the host cells or in the environment. A plethora of these virulence determinants have become the attractive targets for the development of therapeutics inhibiting bacterial adhesion, biofilm formation, assembly or function of secretion systems, virulence factors themselves, their biosynthetic pathways, or regulatory systems such as quorum sensing (QS) (Rasko & Sperandio, 2010). Antivirulence agents are aimed to disarm bacteria and reduce their pathogenicity allowing to host immune system and its normal microbial flora to prevent pathogen colonization and clear the established infection.

The antivirulence approach has several advantages over traditional antibiotic therapy such as expanded repertoire of pathogen targets, reduced selective pressure which decreases possibility to develop drug resistance, and more targeted action causing less damage to commensal microflora, which is a growing concern in the frequent or long-term use of antibiotics (Ruer, Pinotsis, Steadman, Waksman, & Remaut, 2015).

According to the World Health Organization, due to the increased occurrence of MDR strains, the introduction of new therapeutics is urgently needed to fight infections caused by MDR *Pseudomonas aeruginosa* that is classified as one of the pathogens of critical priority.

25.2 *P. aeruginosa* infections

The most significant species of Gram-negative genus *Pseudomonas* for human and veterinary medicine is opportunistic pathogen *P. aeruginosa* (Gellatly & Hancock, 2013; Westman, Matewish, & Lam, 2010). It can be part of the transient microbiota present on the skin and mucous membranes of healthy animals and humans, but it can also lead to infections and serious diseases due to skin and mucous membranes discontinuation (catheterization or patient intubation, wounds caused by burns, surgical wounds), immunodeficiency (cystic fibrosis, cancer, AIDS), as well as changes in saprophytic microbiota due to the use of antibiotics (Lister, Wolter, & Hanson, 2009). In animals, this species causes a whole range of infectious diseases mostly respiratory tract inflammation, cystitis, mastitis, endometritis, chronic wound infection and otitis, which in adult animals are not accompanied by a high mortality rate (Westman et al., 2010).

P. aeruginosa is often found in hospitals on sanitary, medical and cleaning equipment (Kerr & Snelling, 2009). The most common ways of transmitting strains to patients in hospitals are inhalation of aerosols from sources of contaminated water, aspiration of contaminated water as well as direct and indirect contacts (Blanc, Francioli, & Zanetti, 2007; Streeter & Katouli, 2016; Trautmann, Lepper, & Haller, 2005).

The pathogenesis of pseudomonal infection involves colonization and adherence of bacteria in tissues and organs, bacterial multiplication and local infection, and systemic infection (Westman et al., 2010). At the beginning of infection *P. aeruginosa* uses flagella and pili type IV to colonize the tissue. Lipopolysaccharides (LPS) from the bacterial cell wall also have important role being responsible for bacterial binding to the host cells and protection from the immune response of the host (Westman et al., 2010).

Further development of infection is followed by multiplication of bacteria at infection site and the production of chemical signals called autoinducers (AIs) which are part of QS, bacterial cell-to-cell communication system. When bacterial population reaches a critical number, AIs become positive regulators of virulence factors expression, responsible for impairment of the membrane integrity, cytotoxicity, and inactivation of the immune mechanisms of the host (Kipnis, Sawa, & Wiener-Kronish, 2006). When bacteria manage to overcome the immune system of the host, the infection spreads and becomes systemic. Described stages of the pathogenesis of *P. aeruginosa* infection correspond to acute infections. During the development of a chronic infection, bacteria adapt to new conditions and change their lifestyle by switching from the planktonic to life in the form of biofilm (Lee & Yoon, 2017).

P. aeruginosa produces numerous virulence factors and secondary metabolites. These factors can be grouped as factors associated with the bacterial cell (flagella, pili and LPS), factors secreted outside the cell (exotoxin A, proteins, elastases, pyocyanin, pyoverdine, rhamnolipids), and type I, II, and III secretion systems. The development of an acute or chronic infection is closely related to numerous regulatory systems that collect signals from the environment and modulate the expression of the virulence genes. The ability to form a biofilm is a virulence determinant per se, which combines the structures necessary for the mobility of the bacteria, the ability of secretion, and the branched network of intercellular communication.

P. aeruginosa possesses a single polar flagellum, filamentous structure that allows bacteria to perform swimming motility (Gellatly & Hancock, 2013). Pili type IV are filamentous cellular structures located on the cell membrane and they are considered the most important adhesive elements of *P. aeruginosa* (Gellatly & Hancock, 2013), also required for twitching motility. Together flagella and pili participate in the third type of motility called swarming, which is regulated by QS through control of production and secretion of rhamnolipids that have the function of biosurfactants (Köhler, Curty, Barja, van Delden, & Pechère, 2000). Rhamnolipids play important role in all stages of biofilm

formation (Hall-Stoodley, Costerton, & Stoodley, 2004) and can induce necrosis of polymorphonuclear leukocyte (Tolker-Nielsen, 2014). *P. aeruginosa* produces nonhemolytic (PlcN) and hemolytic (PlcH and PlcB) phospholipases C, with latter responsible for direct lysis of erythrocytes (Westman et al., 2010). It also produces alkaline proteinase (aeruginolysin), proteinase IV, elastases LasA and LasB, which are required for the host tissue invasion (Gellatly & Hancock, 2013). *P. aeruginosa* produces siderophores pyoverdine and pyochelin that have the role in collecting iron from the host tissue (Schalk & Guillon, 2013).

P. aeruginosa secondary metabolite hydrogen cyanide (HCN) potently inhibits cytochrome c oxidase and other metalloproteinases, and suppresses aerobic respiration causing death of infected host cells (Blumer & Haas, 2000).

P. aeruginosa produces several phenazines among which pyocyanin (5-methyl-1-hydroxyphenazine) is the only one important for the pathogenesis of pseudomonal infection (Parsons et al., 2007). Pyocyanin induces cytotoxicity in different hosts by causing formation and release of reactive oxygen species (ROS) (Lau, Hassett, Ran, & Kong, 2004; Liu & Nizet, 2009; Winstanley & Fothergill, 2009). It can also chelate iron (Cox, 1986) and exhibit antimicrobial activity (Baron & Rowe, 1981).

The type III secretion system (T3SS) is a multiprotein complex used by bacteria to transfer effector proteins (cytotoxins) into the host cytoplasm (Westman et al., 2010). This secretion system has critical role during acute infection, while its expression decreases as the infection becomes chronic (Gellatly & Hancock, 2013; Hauser, 2009).

Exotoxin A is another important virulence factor that upon entering the cytoplasm of host cell leads to inhibition of the protein synthesis and apoptosis of the host cell (Michalska & Wolf, 2015).

The ability to form biofilms is the central virulent characteristics of *P. aeruginosa* (Milivojevic et al., 2018). When crossing from planktonic form to biofilms *P. aeruginosa* loses motility and virulence factors important for acute infection, and gain protection against phagocytosis, oxidative stress, and the action of antibiotics (Moradali, Ghods, & Rehm, 2017). The importance of *P. aeruginosa* biofilm for human and veterinary medicine is primarily seen in the ability of this bacterium to cause chronic infection (Tolker-Nielsen, 2014; Westman et al., 2010). However, equally important is the fact that this bacterium can form a biofilm on all nonliving surfaces (Lee & Yoon, 2017). Biofilms are highly organized microbial communities enclosed in self-produced extracellular polymeric substances (EPS), which consists of polysaccharides, lipids, nucleic acids, and proteins (Hall-Stoodley et al., 2004). Biofilm confers an extreme capacity for persistence against phagocytosis, oxidative stress, restrictions in nutrients and oxygen, accumulation of metabolic waste, interspecies competition, and most importantly, conventional antibacterial agents (Moradali et al., 2017). Within biofilms bacteria exchange nutrients, metabolic products, and genetic material (Donlan, 2002). This genetic exchange enables spreading of antibiotic resistance genes.

25.3 Therapeutic options against *P. aeruginosa* infections

Due to the spread of MDR *P. aeruginosa* strains therapeutic options for these infections are severely limited and they remain life-threatening complication (Streeter & Katouli, 2016). The options to prevent the spread of infections caused by resistant strains of *P. aeruginosa* include maintaining hygiene, enhanced monitoring, and careful, correct and targeted use of antibiotics. In addition, the development of adequate vaccines, which would be applied to the most vulnerable groups, would help reduce the number of infections, and in the case of cystic fibrosis patients extend and improve the quality of life.

The appearance of nosocomial *P. aeruginosa* isolates resistant to all antipseudomonal antibiotics tested has recently been reported (Muimhneacháin, Reen, O'Gara, & McGlacken, 2018). Beside the efforts to discover new antibiotics, current research is also focused on the development of other types of compounds with bactericidal activity. Antibacterial activity of metal ions is known for a long time but still continues to attract the attention of researchers (Klasen, 2000). Metals exhibit antibacterial activity by interfering with the enzyme function, ROS production, impairing the integrity of membrane, obstructing the entry of nutrients into the bacterial cell, and genotoxicity (Lemire, Harrison, & Turner, 2013). Complexes of silver (I) exhibit more desirable therapeutic characteristics than other metals due to higher antibacterial activity and less toxicity to mammalian cells (Lemire et al., 2013). These complexes also show inhibitory activity against *P. aeruginosa* biofilms (Bjarnsholt et al., 2007; Glisic et al., 2016; Savic et al., 2016). As with antibiotics, resistance to metal ions can be developed.

Other therapeutic options against *P. aeruginosa* are focused on targeting the key factors that contribute to the establishment and progress of infection such as adhesion (Kolomiets et al., 2009) and biofilms (Koo, Allan, Howlin, Stoodley, & Hall-Stoodley, 2017), T3SS (Anantharajah, Mingeot-Leclercq, & Van Bambeke, 2016), and global virulence regulatory system QS (Soukarieh, Williams, Stocks, & Camara, 2018).

25.3.1 Inhibitors of adhesion and biofilms

Biofilm formation and persistence contribute to the bacterial pathogenicity, tolerance, and resistance to antibiotics; therefore different strategies have been developed for their prevention or dispersion of mature biofilms (Bjarnsholt, Ciofu, Molin, Givskov, & Hoiby, 2013).

Premature biofilms can be treated effectively with antibiotics if diagnosed early during infection. However, the diagnosis of premature biofilms is often difficult and ineffective, which is the main reason for the occurrence of clinical conditions accompanied by mature biofilms. Preventive approaches can be applied only as prophylactics, such as in the prevention of biofilm formation on catheters or implants. One such approach is the usage of antiinfective materials with antimicrobial or antibiofilm activities (Aleksic et al., 2017; Costoya et al., 2019; Zizovic et al., 2018). Inhibition of biofilm formation can be achieved by using antibiofilm agents that: (1) prevent microbial attachment to surfaces (Aleksic et al., 2017), (2) modulate bacterial cell-to-cell communication by interfering with QS systems, responsible for biofilm formation (Perez-Perez, Jorge, Perez Rodriguez, Pereira, & Lourenco, 2017), (3) hamper regulatory mechanisms, like nucleotide second messenger signaling systems (Christensen et al., 2013), or (4) disrupt the biofilm architecture (Parrino et al., 2019).

If biofilm prevention cannot be achieved, then approaches dealing with mature biofilms have to be applied and they include mechanical or surgical removal of biofilms if formed on accessible surfaces, biofilm weakening or their disruption. Promising strategies for biofilm weakening and dispersion comprise utilization of effective antibiofilm molecules such as surfactants (reduce bacterial adhesiveness and biofilm maturation) (Aleksic et al., 2017), small molecules that inhibit EPS synthesis (Van Tilburg Bernardes, Charron-Mazenod, Reading, Reckseidler-Zenteno, & Lewenza, 2017), enzymes which degrade matrix of biofilm (DNases targeting extracellular DNA, glycoside hydrolase that hydrolyses the extracellular polysaccharide poly-*N*-acetylglucosamine, and alginate lyase which degrades alginate in mucoid biofilms) (Koo et al., 2017) or molecules influencing c-di-GMP levels (Ma, Yang, Pu, Peti, & Wood, 2011). Inhibition of biofilm matrix production or its disruption releases planktonic bacteria, which could be cleared by phagocytosis or effectively killed by antibiotics. The effective strategies to combat bacterial biofilms and the current results have extensively been reviewed elsewhere (Parrino et al., 2019; Roy, Tiwari, Donelli, & Tiwari, 2018).

25.3.2 Inhibition of Type III secretion system

T3SS is one of the main virulence factors of Gram-negative bacteria, generally associated with acute infections. This system comprises syringe-like export machinery used to inject toxins (effectors) directly from bacterial cytosol into the host cell (Deng et al., 2017). The effectors are delivered into the host cytosol owing to combined action of (1) a secretion apparatus that transports them through the bacterial membranes and (2) a translocon, which enables translocation through the membrane of the host cell (Mattei et al., 2011). Upon activation by the contact with eukaryotic cells, T3SS interferes with signal transduction (Mounier et al., 2012) causing cytotoxicity mediated by effectors (Senerovic et al., 2012), and altering immune responses (Guignot & Tran Van Nhieu, 2016).

The type III secretion systems of pathogens have numerous structural characteristics in common suggesting that a single class of molecules may inhibit T3SS of diverse bacterial species (Anantharajah et al., 2016). When the T3SS is rendered nonfunctional, the majorities of bacteria that express T3SS become benign and are easily cleared by the host immune system. Importantly, T3SS is not found in commensal bacteria. All of these make T3SS an attractive antivirulence target with broad-spectrum application.

Different approaches can be employed to inhibit T3SS activity or prevent the consequences of its activation and they include targeting (1) regulation of T3SS expression, (2) functionality of the injecting apparatus, (3) its ATPase activity, (4) different structural parts of secretion apparatus, or (5) T3SS effector proteins or their chaperones. Up to today, several structurally different inhibitors of T3SS have been identified mostly by screening and not by rational design towards a specific target. Strategies for inhibition of T3SS, as well as identified inhibitors have recently been reviewed (Anantharajah et al., 2016).

25.3.3 Quorum sensing system as antivirulence target

Quorum sensing is a global regulatory system of bacterial virulence with no homologous components in humans, thus its inhibition is considered the most attractive strategy for the development of antivirulence agents.

Quorum sensing comprises production of AIs, their accumulation, detection, and population-wide response involving regulation of the virulence genes expression. As bacterial population density increases AIs accumulate in the

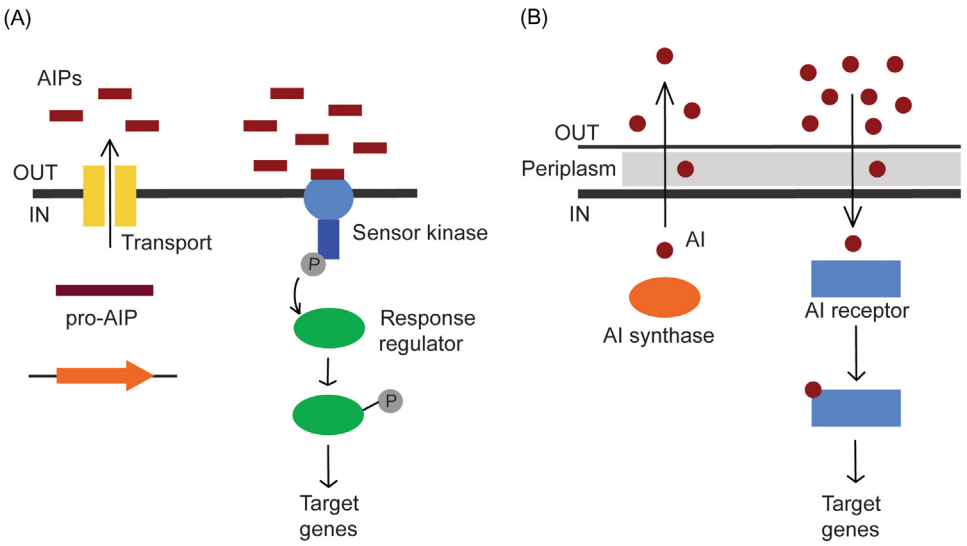

FIGURE 25.1 Bacterial quorum sensing circuits. (A) Gram-positive bacteria produce short oligopeptides (AIPs) for communication, whereas (B) Gram-negative bacteria communicate via small molecules (AI, autoinducer).

environment. At high concentrations, they bind to specific receptors and activate target genes among which are AIs synthases leading to autoinduction of QS circuits. Processes under control of QS include sporulation, bioluminescence, competence, antibiotic production, and virulence factors production and biofilm formation (Bhardwaj, Vinothkumar, & Rajpara, 2013).

Gram-positive and Gram-negative bacteria use different types of signaling molecules. Gram-positive bacteria produce short oligopeptides as AIs (AIPs), in most cases synthesized as a longer peptide precursor that are upon secretion modified by a specific transporter (Fig. 25.1A). At high concentration AIPs bind to a related membrane-bound two-component histidine kinase receptors. Usually, the binding of signal molecule activates kinase activity of the receptor resulting in autophosphorylation, which in turn transfers phosphate to a corresponding cytoplasmic response regulator. Upon phosphorylation response regulator activates transcription of the genes under QS regulation (target genes). In certain cases, AIPs are transported back into the cell cytoplasm where they modify activity of transcription factors, which then in turn change target genes' expression (Rutherford & Bassler, 2012).

Signaling molecules of Gram-negative bacteria are small molecules such as acyl homoserine lactones (AHLs), alkyl-quinolones, thiazole compounds, α-hydroxyketones, and diffusible signal factors (fatty acid-like compounds). These AIs are synthesized in a single reaction or through a series of enzymatic reactions from fatty acids, anthranilate and S-adenosylmethionine (SAM). When the concentration of AIs reaches threshold, they bind to cytoplasmic receptors that are transcription factors and regulate expression of the target genes under QS regulation (Fig. 25.1B). In certain cases, AIs are sensed by two-component histidine kinase receptors similarly to those of Gram-positive bacteria.

25.4 Quorum sensing in *P. aeruginosa*

QS plays a central role in regulating the expression of virulence genes in *P. aeruginosa* and consists of four interconnected signaling pathways: two LuxI/LuxR-type QS systems, Pseudomonas Quinolone Signal (PQS), and integrated QS system (IQS), all organized in a hierarchical manner (Fig. 25.2) (Papenfort & Bassler, 2016).

The first discovered bacterial signaling molecules were AHLs used by Las and Rhl systems. Since AHLs are AIs in numerous Gram-negative bacteria, they remain the most-studied bacterial communication molecules (Jimenez et al., 2012). They comprise homoserine lactone moiety linked via peptide bond to fatty acids, which can vary in length and substitution (Fig. 25.3). The Las system use N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) that is synthesized by LasI synthase, the homolog of LuxI, and sensed by the cytoplasmic LuxR homolog, LasR (Pearson et al., 1994). Rhl system uses N-butanoyl homoserine lactone (C4-HSL) as signaling molecule (Pearson, Passador, Iglewski, & Greenberg, 1995), which is generated by the second LuxI homolog called RhlI synthase and recognized by a second LuxR homolog the transcriptional regulator RhlR. The complex LasR-3-oxo-C12-HSL triggers expression of elastases, exotoxin A, alkaline protease, *lasI* itself, and *rhlR*. RhlR-C4-HSL complex stimulates rhamnolipids' production and biofilm formation, but represses PQS system and genes involved in assembly and function of the T3SS.

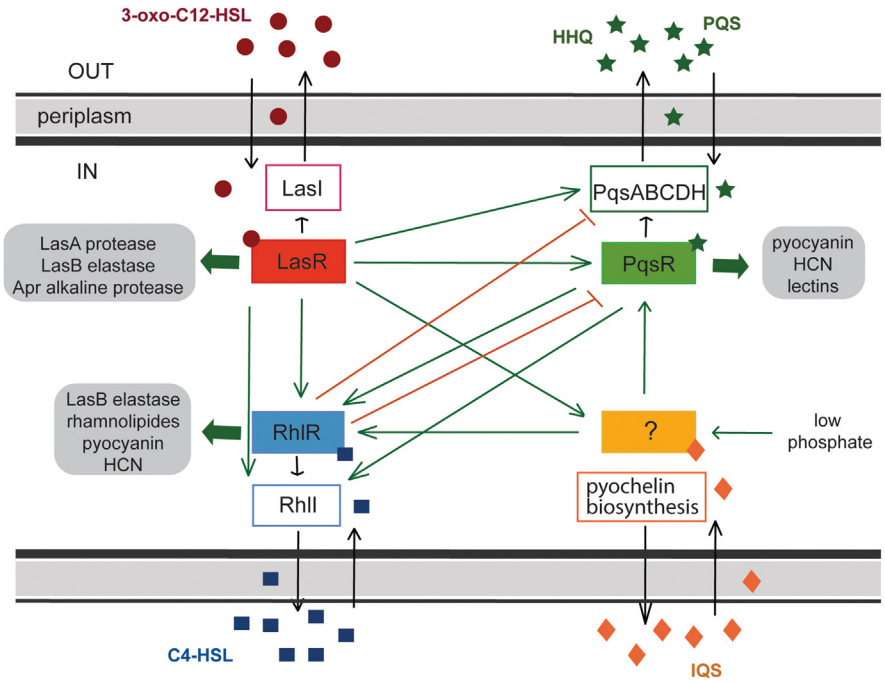

FIGURE 25.2 Hierarchy of quorum sensing signaling pathways in *P. aeruginosa*. *Arrows* represent induction and *blunt bars* represent downregulation of target genes.

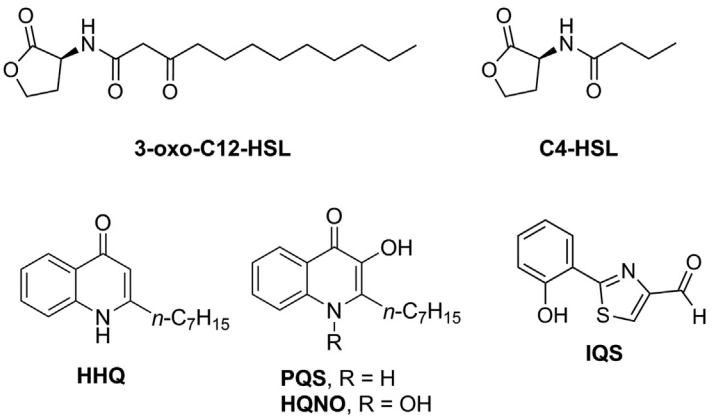

FIGURE 25.3 Structures of autoinducers commonly used by *P. aeruginosa*.

Beside C4-HSL and 3-oxo-C12-HSL other AHLs, such as 3-oxo-C14-HSL and 3-oxo-C10-HSL, were isolated from the supernatants of *P. aeruginosa* cultures but in lower concentrations (Charlton et al., 2000). *P. aeruginosa* also produces diketopiperazines (DKPs), small diffusible signaling molecules which at high concentrations can activate AHL signaling pathways (Holden et al., 1999). Cyclo(Ala-L-Val) and cyclo(L-Pro-L-Tyr) have been identified in the supernatants of *P. aeruginosa*. Diketopiperazines can interfere with the QS systems of different bacteria, probably through binding to the receptors of LuxR family, either as activators or antagonists of AHLs. DKPs are also produced by animals and higher plants and they show wide spectrum of biological and pharmacological activities. Most likely the DKPs do not function as cell-to-cell signals per se, but they might be involved in modulation of prokaryotic−eukaryotic interactions (Holden, Swift, & Williams, 2000).

P. aeruginosa synthesizes over 50 different alkylquinolines (AQs) (Deziel et al., 2004) of which 2-heptyl-3-hydroxy-4-quinolone (PQS) and its immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ) are most closely associated with QS signaling (Fig. 25.3). Synthases PqsA, PqsB, PqsC, PqsD, PqsE, and PqsH produce PQS and HHQ that can be both detected by the receptor PqsR (LysR-type receptor also known as MvfR) (Gallagher, McKnight, Kuznetsova, Pesci, & Manoil, 2002; Xiao et al., 2006). Formation of complex of PqsR with either PQS or HHQ initiates the transcription of the *pqsABCDEH* operon leading to the synthesis of PQS and HHQ. In addition, PqsR triggers

expression of *rhlI* and the synthesis of several virulence factors such as pyocyanin and hydrogen cyanide, as well as biofilm formation. The expression of *pqsR* is positively regulated by LasR, but repressed by RhlR (Papenfort & Bassler, 2016). Until today AQ signaling has been found only in *P. aeruginosa* and certain *Burkholderia* and *Alteromonas* species (Diggle et al., 2006; Vial et al., 2008).

Almost 10% of the *P. aeruginosa* genome is under control of QS (Schuster & Greenberg, 2006), and fine-tuning of QS system is established by multiple regulators such as AlgR, DksA, GidA, PhoR/B, PhrS, PrrF, QscR, RelA, RpoN, RpoS, RsaL, RsmA, RsmY, RsmZ, Vfr, VqsM, and VqsR, which all secure optimal precision of virulence factors production (Rutherford & Bassler, 2012).

25.4.1 Inhibition of *P. aeruginosa* quorum sensing signaling

Interference with QS signaling leads to repressed production of virulence factors and consequently less severe infections that could be more easily cleared by immune system of the host. Additionally, reduced ability to form biofilm may increase bacterial susceptibility to antibiotic agents, improving the effectiveness of antibacterial treatments.

Inhibition of QS could be achieved by three different mechanisms: (1) inhibiting the signal generation, (2) inactivating the signal molecules (enzymatically or by scavenging of signaling molecules by antibodies or other macromolecules), and (3) competing with AIs for binding to receptors. Application of each of these strategies should lead to disruption of bacterial communication and is expected to attenuate virulence.

25.4.1.1 Inhibition of signal generation

While short chain AHLs and PQS can freely diffuse, long-chain AHLs and HHQ require efflux pumps for transport (Lamarche & Deziel, 2011; Pearson, Van Delden, & Iglewski, 1999). Thus signal generation could be blocked either by targeting enzymes involved in AIs synthesis or efflux pumps that enable transport and accumulation of signaling molecules inside the cells.

AHLs are synthetized by AHL synthases of the LuxI or AinS family starting from SAM and acyl-carrier protein (ACP) (Gilson, Kuo, & Dunlap, 1995; Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999). As such, AHL synthesis could be inhibited by repression of SAM biosynthesis, interfering with acyl-ACPs generation, or inactivation of the synthase itself. Inhibition of AHL production is the least investigated strategy so far. Several substrate analogs, such as l/d-*S*-adenosylhomocysteine, butyryl-SAM, holo-ACP, and sinefungin that have been identified as blockers of AHL production in vitro (Parsek et al., 1999), have not been tested on bacteria in vivo. One of the explanations is that the inhibitors of AHL biosynthetic pathway are expected to interfere with the central pathways of amino acid and fatty acid metabolism (Rasmussen & Givskov, 2006).

Most of the genes required for AQ biosynthesis are located in the *pqsABCDE* operon (Heeb et al., 2011). PqsA, a carboxylic acid-CoA ligase, transforms anthranilic acid to anthraniloyl-CoA, which is then, in a reaction catalyzed by PqsD, condensed with malonyl-CoA forming 2-aminobenzoylacetyl-CoA (2-ABA-CoA). 2-ABA-CoA is converted into 2-aminobenzoylacetate (2-ABA) by thioesterase activity of PqsE. Through the condensation of 2-ABA and octanoyl-coenzyme A HHQ is formed via the PqsBC heterodimer. Under aerobic conditions, monooxygenase PqsH hydroxylates HHQ at C(3) position in the last step of PQS biosynthesis (Heeb et al., 2011). PqsL, a second monooxygenase, together with the *pqsABCD* gene products is required for the synthesis of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and related N-oxides. Inactivation of PqsA, PqsB, or PqsD completely blocks HHQ and PQS biosynthesis in *P. aeruginosa* as well as PQS regulated virulence factors expression, thus these enzymes are considered the most promising targets for quenching of PQS signaling.

25.4.1.2 Inactivation of autoinducers

Autoinducers inactivation can be achieved either by degradation of signaling molecules (strategy called quorum quenching, QQ) or scavenging by antibodies (Rasmussen & Givskov, 2006). The methods for degradation of AIs include chemical and enzymatic degradation, as well as metabolic destruction of AHLs by certain bacteria, which are able to use them as sole source of energy, carbon and nitrogen.

The simple way to degrade AHL signaling molecules is to increase environmental pH above 7, which causes reversible lactonolysis followed by inhibition of QS-regulated genes and virulence factors expression. Additionally, the increase of the temperature accelerates lactone ring opening but this is limited to AHLs with side chains longer than four carbons (Yates et al., 2002).

Enzymatic degradation of AHLs can be attained by lactonases, acylases, and oxidoreductases. AHL lactonases hydrolyze the ester bond in the homoserine lactone ring producing the acyl homoserine molecule (Dong, Gusti, Zhang, Xu, & Zhang, 2002). Lactonases exhibit broad AHL substrate specificity as a result of conserved homoserine lactone ring in different AHLs, and nonspecific interactions of variable acyl chain within the active-site cavity of the enzyme (Liu et al., 2008). The downside of the lactonolysis reaction is its reversibility at acidic pH, when a ring-opened AHL molecule spontaneously undergoes ring formation regardless of the method by which it was opened (chemical or enzymatic).

AHL acylases nonreversibly hydrolyze the acyl-amide bond between the acyl tail and lactone ring of AHLs, resulting in the release of a fatty acid chain and a homoserine lactone moiety (Lin et al., 2003). Acylases exhibit substrate specificity based on the acyl chain length of the AHL and substitution on the third position of the chain due to a constrained binding pocket in the enzyme that must adapt structurally upon interaction with the ligand (Bokhove, Nadal Jimenez, Quax, & Dijkstra, 2010).

Oxidoreductases inactivate AHLs by oxidation or reduction of the acyl side chain (Uroz et al., 2005). They are the least investigated AHL-targeting enzymes identified so far.

Hod dioxygenase hydrolyzes PQS into N-octanoyl anthranilic acid (Pustelny et al., 2009). Purified Hod enzyme inhibits PQS signaling in cultures of *P. aeruginosa*, but loses its activity as a result of proteolysis by secreted bacterial proteases.

Sequestering autoinducers with antibodies is highly specific method for deactivation of signaling molecules (Palliyil, Downham, Broadbent, Charlton, & Porter, 2014). It provides efficient inhibition of QS signaling and prevents cytotoxicity and immunosuppression elicited by AHLs in the host cells (Kaufmann, Park, Mee, Ulevitch, & Janda, 2008).

25.4.1.3 Competition for Binding to Receptors

Quorum sensing receptors are major regulators controlling expression of plethora of virulence factors including biofilm formation and thus QS inhibitors, which target these receptors, are the focus of extensive investigation. With the aim to develop potent receptor blockers, numerous analogs of native signaling molecules have been synthetized. Modifications in native signals are made in such a way to preserve the interaction of signal with the receptor while interrupting downstream signaling. Generated signal−receptor complexes further inhibit binding of the native signal. The structures of known inhibitors have been altered in order to improve their inhibitory activities or change their target specificities.

Quorum sensing antagonists have been obtained either by rational design or screening of random, structurally nonrelated, compound libraries. High-throughput approach coupled with computer-aided screens of small molecules have significantly extended the list of known QS modulators and have increased the number of structural scaffolds that could be used as bases for the development of novel QS inhibitors. It is noteworthy to mention that even the small structural changes within the AIs structure could change the molecule's functionality leading to generation of analogs with either agonistic or antagonistic activity. Although extensive structure−activity relationship (SAR) and molecular modeling studies have been performed with the aim to identify the most potent inhibitors of QS receptors, the precise binding requirements which would distinguish agonists from antagonists remain unknown and seems to vary among individual receptors (LaSarre & Federle, 2013).

The AHL analogs differ in design of their acyl side chain or the lactone ring or in both of these moieties. The inhibitors of AHL receptors have been extensively reported (Geske, O'Neill, & Blackwell, 2008; Welsh & Blackwell, 2016) and only the recent advances are discussed within this chapter.

The design of PqsR inhibitors involves modifications of alkyl chains or substitutions of benzene ring within PQS and HHQ (J. Hodgkinson, Bowden, Galloway, Spring, & Welch, 2010; Lu et al., 2012). Detailed SAR analyses of different PqsR ligands enabled identification of structural characteristics for distinguishing agonists from antagonists and have made rational design of PqsR inhibitors more feasible (Klein et al., 2012).

25.4.2 Natural products as quorum sensing inhibitors

Various bacteria can inhibit QS of other bacteria by degrading their signaling molecules using QQ enzymes. Most of bacteria capable to inactivate AHLs contain one or two enzymes exhibiting this activity. The exceptions are *P. aeruginosa*, which has multiple AHL acylases, and *Rhizobium* sp. strain NGR234, known to carry at least five different AHL-degrading loci (LaSarre & Federle, 2013). Soil bacterium *Arthrobacter nitroguajacolicus* produces Hod dioxygenase that hydrolyzes PQS (Pustelny et al., 2009). Soil bacteria have been well known for their ability to produce secondary

metabolites with antibiotic, antifungal, antiviral, antitumor, and immune-suppressive activities (Genilloud, 2017), but recently several soil isolates have been identified to produce small molecules with anti-QS activity (Du et al., 2018; Hassan, Shaaban, Abdel Bar, El-Mahdy, & Shokralla, 2016; Polkade, Mantri, Patwekar, & Jangid, 2016). One such example is soil isolate *Streptomyces coelicoflavus*, which produces 1*H*-pyrrole-2-carboxylic acid that inhibits pyocyanin, protease, and elastase production in *P. aeruginosa* without affecting its viability (Hassan et al., 2016).

Eukaryotes like algae, fungi, or plants live together with both beneficial and pathogenic bacteria so they have developed various mechanisms to interact with them. The first identified QS inhibitors were halogenated furanones isolated from the marine red alga *Delisea pulchra* (Givskov et al., 1996). These halogenated furanones are structurally similar to AHLs, having furan instead of a homoserine lactone ring. One of the most-studied natural compounds is (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone, which inhibits AHL-regulated virulence factors in *P. aeruginosa* (Aleksic et al., 2018; Hentzer et al., 2002).

Fungi are known to produce secondary metabolites such as antibiotics. Penicillin producer *Penicillium* sp. also produces anti-QS compounds patulin and penicillic acid (Rasmussen et al., 2005). Opportunistic fungal pathogen *Candida albicans*, which is often found together with *P. aeruginosa* in clinically relevant mixed infections, produces signaling molecule sesquiterpene farnesol that inactivates PqsA and thus inhibits PQS production (Cugini et al., 2007).

Plants' secondary metabolites (phytochemicals) are the major reservoir of chemical diversity and valuable bioactive elements of current pharmaceuticals. Different water and organic extracts have been tested for numerous bioactivities including antimicrobial, anticancer, antidiabetic. Hundreds of reports also demonstrate potent anti-QS activity of plants' extracts against different bacterial species (Silva, Zimmer, Macedo, & Trentin, 2016). QSI activity for most of these extracts has been described based on their effect on bacterial virulence factors expression and biofilm formation, but the active components and their mechanism of action remained to be elucidated (Hossain et al., 2015; Husain et al., 2017; Yin et al., 2015). However, there are still a lot of plant extracts with identified bioactive components and explained mechanism of action (Koh et al., 2013; Silva et al., 2016). Plant-derived natural products which show activity against QS and/or biofilm formation include several classes of compounds including alkaloids, fatty acids, organosulfurs, some aliphatic compounds, cyclic compounds, phenolics, steroids, terpenoids and their derivatives. Most of these compounds inhibit AHL-regulated gene expression (Borges et al., 2014; Myszka et al., 2016; Sarabhai, Sharma, & Capalash, 2013; Singh et al., 2009) most likely through direct binding with LasR, but some of them such as phenolic acids, ellagic and ferulic acids, can also interfere with PQS signaling pathway by competitive binding to PqsR (Aleksic et al., 2018).

Essential oils (EOs) are complex mixtures of volatile compounds extracted from plants, which show different bioactivities including antimicrobial, antiinflammatory, antioxidant, anticancer, antiinsecticidal, and other properties (Edris, 2007). Rose, geranium, lavender, rosemary, pompia, and grapefruit EOs showed the ability to modulate bacterial QS signaling and to inhibit biofilm formation (Alves, Duarte, Sousa, & Domingues, 2016; Kalia et al., 2015; Kerekes et al., 2013; Pekmezovic et al., 2016; Vikram, Jesudhasan, Jayaprakasha, Pillai, & Patil, 2010). Citrus EOs are effective against bacterial, fungal, or mixed species biofilms. We have shown that grapefruit and pompia EOs inhibit AHL signaling pathway in *P. aeruginosa*, as well as formation of single and mixed species biofilms composed of *P. aeruginosa* and different medically relevant fungi. Lemon EO inhibits both monomicrobial and mixed biofilms formed by *Escherichia coli* and *Bacillus cereus* (Kerekes et al., 2013; Vikram et al., 2010).

25.4.3 Synthetic compounds with quorum sensing inhibitory activity

The number of chemically synthesized molecules that inhibit bacterial communication rises, but they are mostly chemically modified naturally-derived QS antagonists (Koh et al., 2013; Silva et al., 2016).

25.4.3.1 Inhibitors of las signaling pathway

AHLs are often used as structural motif for the synthesis of new QSI, since it is expected that modification of structural scaffold of these autoinducers could produce selective inhibitor(s) of bacterial signaling pathway(s). Kalaiarasan et al. described the synthesis of two homologs of *N*-acyl substituted homoserin lactone, **1** and **2** (Fig. 25.4) (Kalaiarasan et al., 2017), selected upon the results of simulation of their docking into active site of LasR. Although chloroderivative was somewhat more active than fluoroderivative, both homologs inhibited biofilm formation of MDR *P. aeruginosa* at 100 μM.

Recently, it has been discovered that α-pyrones are signaling molecules detected by orphan LuxR-type receptors (Brachmann et al., 2013). Therefore a series of pyrone derivatives were tested for their capacity to inhibit binding of

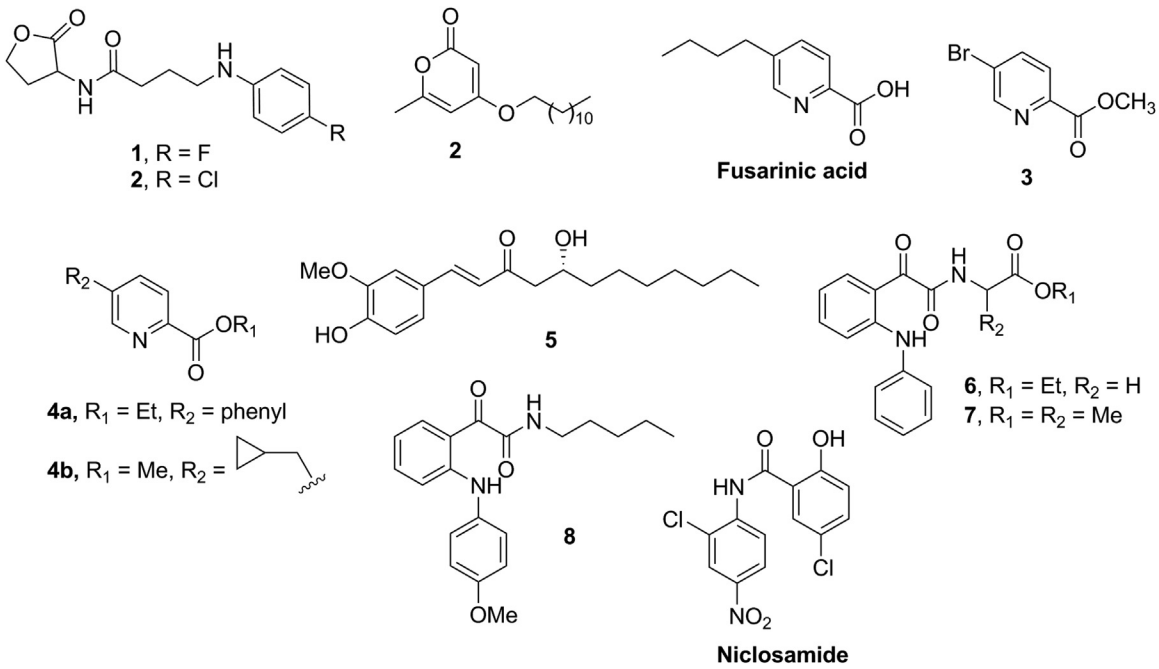

FIGURE 25.4 Structures of Las inhibitors.

3-oxo-C12-HSL to LasR and biofilm formation, as well as their impact on QS-regulated genes in *P. aeruginosa* (Park et al., 2015). The most potent analog proved to be compound **2** that inhibited biofilm formation for 30% at 100 μM concentration and suppressed the expression of 15 selected QS-regulated genes. This compound showed the best binding mode similarity score to LasR. It formed hydrogen bonding with three out of four amino acid residues in comparison with 3-oxo-C12-HSL.

A series of synthetic derivatives of fusarinic acid (Fig. 25.4) (Tung et al., 2017), a natural product isolated from *Fusarium heterosporum* (Yabuta, Kambe, & Hayashi, 1934), was designed to target QS. Among 40 examined derivatives, only one compound (**3**) inhibited *lasB* expression. Further structure modifications giving derivatives **4a** and **4b**, resulted in significantly better inhibition of *luxI*, with $IC_{50} = 12$ μg/mL (**4a**) and $IC_{50} = 14$ μg/mL (**4b**), respectively (IC_{50} is a concentration of the compound at which 50% of its activity is observed).

(*S*)-6-gingerol efficiently reduces production of virulence factors and inhibits biofilm formation (Kim et al., 2009). Detailed SAR study of series of gingerol analogs revealed the importance of the presence of the groups in aromatic part of the molecule capable to form hydrogen bonds with LasR, stereochemistry and rotational rigidity in the middle of the structure, and optimal alkyl side chain length (Choi et al., 2017). The most active compound **5**, an analog of (*R*)-8-gingerol with restricted rotation, demonstrated the strongest affinity to bind LasR and inhibit biofilm formation.

A series of isatine derivatives, a chemotype not related to natural AIs, has been reported as very potent QSIs (Nizalapur et al., 2016). Compounds were obtained via the ring-opening reaction of *N*-aryl isatins with cyclic and acylic amines and amino acid esters. Derivatives **6** and **7** reduced LasR expression by 43%−49%, while compound **8** inhibited production of pyocyanin by 47% at 250 μM. The major impact on the QS inhibitory activity has formation of hydrogen bonding as observed in computational docking studies performed on the LasR receptor.

Repurposing approved drugs to affect a new target or nonrelated disease could be a promising approach in the search for a new lead molecule (Wermuth, 2006). In the study where the library of more than 1000 FDA-approved was screened, niclosamide, anthelmintic drug (Fig. 25.4) was identified as a promising anti-QS compound (Imperi et al., 2013). Niclosamide reduced the level of 3-oxo-C12-HSL through competitive inhibition of LasR. It also affected the production of C4-HSL independently of 3-oxo-C12-HSL inhibition, reduced the level of pyocyanin, elastase, and rhamnolipids, and suppressed swarming motility at low nanomolar concentrations. Nanoparticles of niclosamide could be applied with high efficiency as a saline solution for antivirulence therapy of *P. aeruginosa* lung infections, in vitro and in vivo (Costabile et al., 2015). It has also been reported that niclosamide inhibits biofilm formation in *Xanthomonas oryzae* and interferes with QS related proteins, which makes niclosamide attractive lead for further development (Sahu, Zheng, & Yao, 2018).

25.4.3.2 Inhibitors of Rhl signaling pathway

The reports on development of compounds specifically targeting Rhl system are scarce, and mostly cover synthetic analogs of natural or nonnatural derivatives of homoserine lactones (HSLs).

This type of QSIs are *m*-bromo- and *m*-chloro-thiolactone (**9** and **10**; Fig. 25.5), which inhibit both the production of pyocyanin and biofilm formation with IC_{50} values of 8 and 9 μM, respectively (O'Loughlin et al., 2013). Inhibition of RhlR and LasR depended on the presence of their natural AIs, that is, the activity of **9** was changed from antagonism to partial agonism depending on whether 3-oxo-C12-HSL or C4-HSL was present or not. Derivative **11** (Swem et al., 2009), although better inhibitor of both LasR- and RhlR-regulated transcription comparing to compound **9**, did not inhibit pyocyanin production and it did not affect killing of A549 lung cells by *P. aeruginosa*.

The effects of series of synthetic *N*-acyl (*S*)-homoserine lactones on hierarchical relations within Las−Rhl−PQS QS systems were evaluated, as well as their impact on production of pyocyanin and rhamnolipids (Welsh, Eibergen, Moore, & Blackwell, 2015). It was revealed that the most active RhlR agonists were the strongest inhibitors of pyocyanin production. Derivatives **12** and **13** inhibited pyocyanin production with IC_{50} values of 9.9 and 6.8 μM, respectively. At the same time, they induced production of rhamnolipids by two-fold. In contrast, derivative **14** has proven to be the most active antagonist of RhlR, inhibiting rhamnolipid production by approximately 60%, but stimulated pyocyanin production up to 140%. Derivatives **12** and **13** have shown downregulation of *phzA* and *phzB* genes (involved in pyocyanin synthesis) and *psqA* gene in micromolar concentrations. Nevertheless, they upregulated *rhlA* and *rhlB* genes responsible for rhamnolipid biosynthesis. Interestingly, **12** and **13** exhibited almost no influence on *pqsR*. Detailed SAR study emphasized critical structural requirements for changing of agonist to antagonist activity and vice versa (Boursier et al., 2018). In general, modifications of side chain led to more active agonist, while modifications on lactone moiety led to antagonistic activity. It came out that the most active agonists of RhlR were naturally occurring derivative **15** and cyclobutyl analog **16** (Fig. 25.5) being also the most active LasR antagonists in the library. Transformations on amide group or lactone ring produced new antagonists, with derivatives **17** and **18** as the most active inhibitors of RhlR. The homocysteine thiolactone, C4-HSL analog, is the only example of lactone-like derivative that is more potent than the native ligand.

Based on structural similarity with 8-hydroxyquinolines and natural 2-substituted quinolones that interfere with biofilm formation, a group of 2-aryl-8-hydroxy quinoline esters was investigated as antivirulence and antibiofilm agents (Fig. 25.5) (Qiu et al., 2019). Derivatives **19** and **20** were the most active inhibitors of biofilm formation. They both inhibited *rhlA* expression at 10 μM concentration and showed no effect on *lasB* and *pqsA* gene expression. The assumption that **19** and **20** inhibited QS by interfering with Rhl system was confirmed by showing inhibition of rhamnolipids production and the absence of the influence on pyocyanin level.

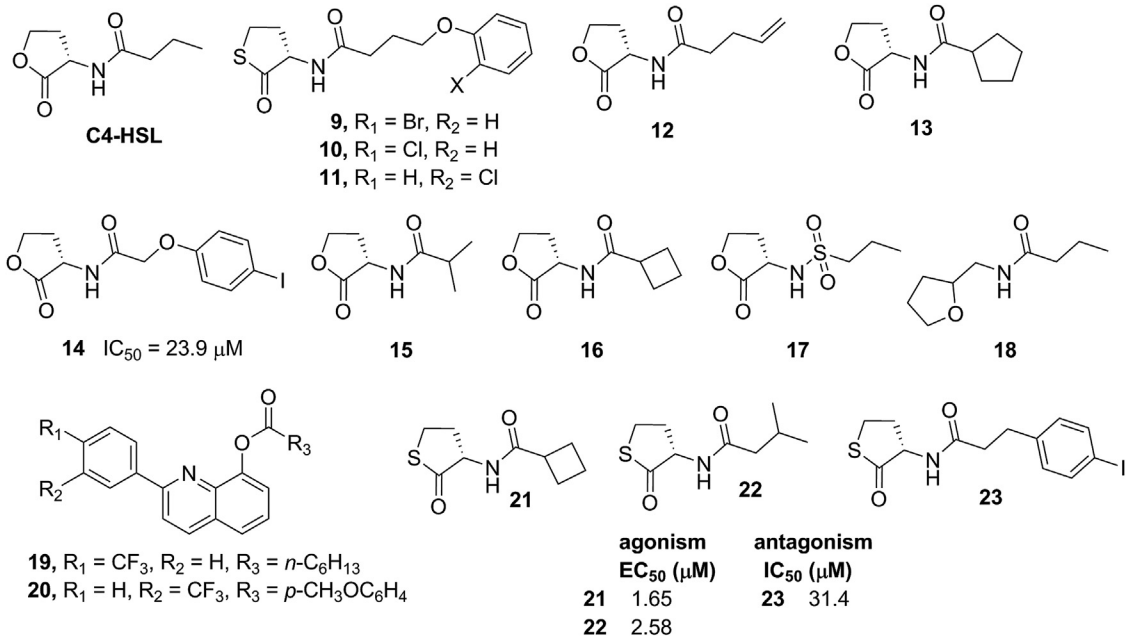

FIGURE 25.5 Structures of Rhl inhibitors.

A series of *S*-homoserine lactones (Boursier, Combs, & Blackwell, 2019) that contain the key structural fragment from previously described RhlR agonists (Basak, Abouelhassan, & Huigens Iii, 2015; Smith, Bu, & Suga, 2003; Soukarieh, Vico Oton, et al., 2018) and antagonists (Ishida et al., 2007; Starkey et al., 2014) was designed. Derivatives **21** and **22** showed the highest agonistic effect at low micromolar concentrations (Fig. 25.5). Derivative **23** showed antagonistic effect with IC_{50} value 31.4 μM.

25.4.3.3 Inhibitors of PQS signaling pathway

The majority of QS synthetic inhibitors have been designed based on the core structures of PQS and its biosynthetic precursor HHQ. While AHL-inspired QS inhibitors have a potential to be broad-spectrum antivirulence agents due to a wide spread usage of AHLs as signaling molecules in different Gram-negative bacteria, quinolone-based compounds are expected to exhibit species-specific activity against *Pseudomonas*, some *Burkholderia* and *Alteromonas* species.

The effects of series of PQS analogs (**24**−**29**) on *pqsA* transcription, PqsR-independent pyoverdine production, and membrane vesicles production have been reported (Hodgkinson et al., 2010). Derivatives **24** and **29** stimulated PQS production, while derivatives **28** and **29** stimulated pyoverdine production. Only chlorosubstituted derivatives (**24**−**27**) stimulated membrane vesicles production (Fig. 25.6).

The presence of hydroxy-group causes agonistic activity of PQS analogs, and this effect was circumvented by synthetizing a series of HHQ analogs (Lu et al., 2012). Modifications in benzene ring and 3-alkyl substituents led to the synthesis of highly potent inhibitors achieving almost complete PqsR inhibition at 5−10 μM concentrations (**30**−**34**; Fig. 25.6). Positions of the substituents on quinoline core have a strong influence on the compounds QSI activity with electron-withdrawing groups being responsible for stronger activity. Derivatives inhibited PqsR at low nanomolar concentration, causing reduced pyocyanin production by 70%. Introduction of polar groups at C(3) position of derivative **34** aiming to increase water solubility of the compound resulted in double fold increase in PqsR inhibition (Lu, Kirsch, et al., 2014). These new derivatives, **35** and **36** completely inhibited activity of PqsR and reduced pyocyanin level by 80% at 5 μM. Their QSI activities were confirmed in in vivo experiments (Lu, Maurer, Kirsch, Steinbach, & Hartmann, 2014).

Ilangovan et al. identified the active place for ligand accommodation and marked the key interactions of the ligand with receptor's amino acid residues required for a good binding (Ilangovan et al., 2013). Using ligand-based design approach quinolon isostere quinazoline (QZN) was designed, as highly potent competitive inhibitor of PqsR with derivatives **37**−**39** being the most active ones (Fig. 25.6). A simple isostere replacement has been shown sufficient to

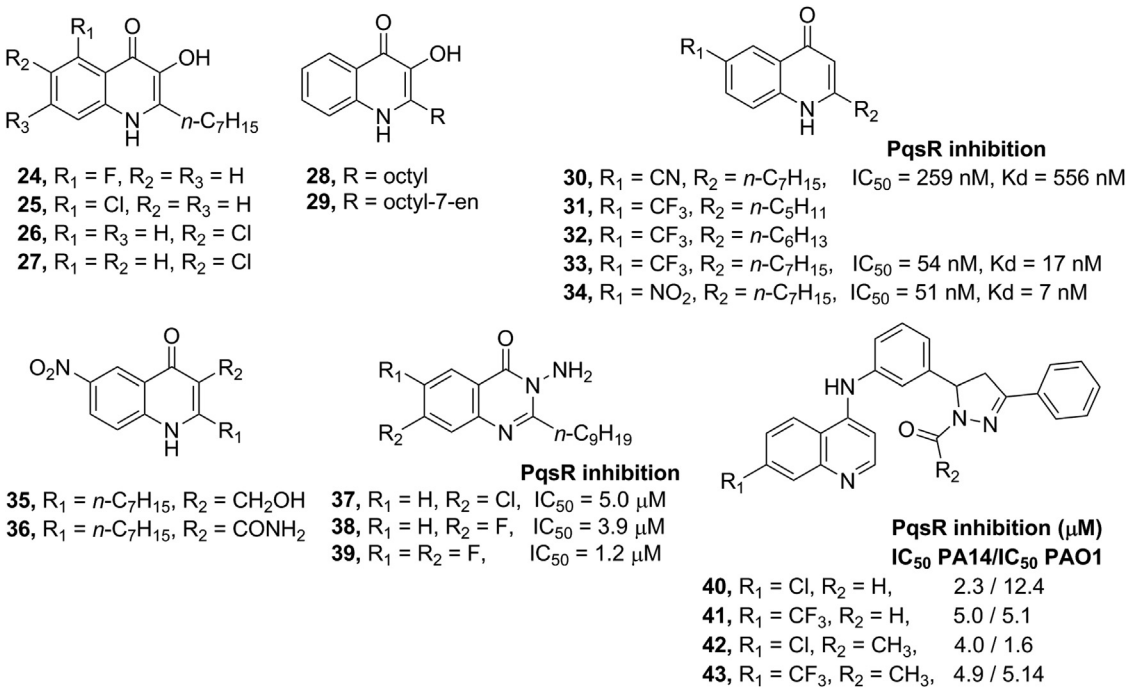

FIGURE 25.6 Selected quinolone structures.

switch a QZN from potent agonist to potent antagonist (OH for NH_2). Derivative **37** antagonized pyocyanin production, virulence gene expression, AQ biosynthesis, and biofilm development.

Quinoline is a well-established pharmacophore used in many drugs including antimalarial drugs chloroquine and quinine. Recently, a series of 4-aminoquinoline derivatives have been described as dual *Plasmodium falciparum* and botulinum neurotoxin inhibitors (Opsenica et al., 2012; Šolaja et al., 2008; Videnović et al., 2014), as well as ligands in complexes with anticancer activities (Nikolić et al., 2015). Following the drug repurposing approach various research groups developed quinoline-based molecules with QSI activities. PqsR antagonists were identified within a series of 31 derivatives of antiprotozoal 4-aminoquinolines (**40**−**43**; Fig. 25.6) inhibiting receptor activity by 70%−85% at 10 μM (Soukarieh, Vico Oton, et al., 2018). Derivatives **40** and **41** inhibited pyocyanin production, while compound **40**, inhibited also HHQ, PQS, and HQNO production, and biofilm formation. The potency of these compounds varied between strains of *P. aeruginosa* (*P. aeruginosa* PA14 vs *P. aeruginosa* PAO1).

PQS, HHQ and some of the 4-quinolone derivatives can modulate interspecies and interkingdom interactions (Fernández-Piñar, Cámara, Dubern, Ramos, & Espinosa-Urgel, 2011; Reen et al., 2011, 2012; Reen, Shanahan, Cano, O'Gara, & McGlacken, 2015), making this chemotype more attractive for further evaluation as QSI. Some of the 4-quinolone derivatives, (Reen et al., 2015) quinoline-aminoalcohols (León, Haeckl, & Linington, 2015), halogenated quinolones (Basak et al., 2015), and 4-aminoquinolines (Aleksić et al., 2017), exhibited inhibitory activity against biofilm formation in *Bacillus atrophaeus*, *Vibrio cholerae*, MRSA, MRSE, VRE, and *Serratia marcescens*.

Many derivatives, structurally unrelated to quinoline, show potent inhibitory activity against PqsR and other targets within PQS signaling pathway. Two hydroxamic acids (**44** and **45**; Fig. 25.7) demonstrated inhibition of PqsR (Klein et al., 2012). The small structural changes like replacing OH-group with hydrogen (**46**; Fig. 25.7) shifted the derivative's activity from antagonist to agonist. The change of Et-group of amide **46** with chlorine in the derivative **47**, led to recovery of inhibitory activity. Compound **48** (Fig. 25.7) bearing 2-amino-oxadiazole as an amide bioisostere group show PqsR inhibitory activity with $EC_{50} = 338.5$ μM, and also suppressed production of HHQ and pyocyanin by half at 250 μM, without effect on PQS levels (Zender et al., 2013).

A series of 1H-benzo[*d*]imidazol-2-thioacetamide, bearing various substituents on amide and 1H-benzo[*d*]imidazole moieties, have recently been reported (Starkey et al., 2014). Derivative **49** (Fig. 25.7) was identified as the most effective inhibitor that reduced PQS, HHQ, and pyocyanin production with IC_{50} at 200, 350, and 300 nM, respectively. In addition, this compound reduced *P. aeruginosa* virulence in mouse burn and lung infection models. Those compounds were the first molecules identified as reducers of the formation of antibiotic-tolerant persister cells in mice. Crystal structure of **49**-PqsR complex revealed that **49** act as a competitive inhibitor of PqsR (Kitao et al., 2018).

Among the first reported inhibitors of HHQ and PQS synthesis were halogenated derivatives of anthranilic acid (Lesic et al., 2007), which targeted PqsA and reduced *P. aeruginosa* virulence in a thermal injury mice model.

PqsD is the key enzyme in the synthesis of HHQ, which makes it an attractive target for developing of PQS inhibitors (Pistorius et al., 2011). Several different series of small molecules with PqsD inhibitory activity were described. Following the ligand-based approach based on anthranilic acid, series of *o*-nitro and *o*-amino aromatic derivatives were investigated as PqsD inhibitors (Storz et al., 2012). Amino derivatives were inactive, while corresponding nitroderivatives **50**−**54** (Fig. 25.8) showed almost complete inactivation of PqsD. By gradual simplification of structures, **54** was identified as the most active compound inhibiting PqsD completely. Furthermore, **54** suppressed HHQ and PQS production and reduced biofilm formation by 77%, 42%, and 38% at 250 μM, respectively.

Another amide derivatives of anthranilic acid proved effective against PqsD (Pistorius et al., 2011). Further improvement of inhibitory activity was achieved with new derivatives (**55**−**58**; Fig. 25.8) (Weidel et al., 2013) which

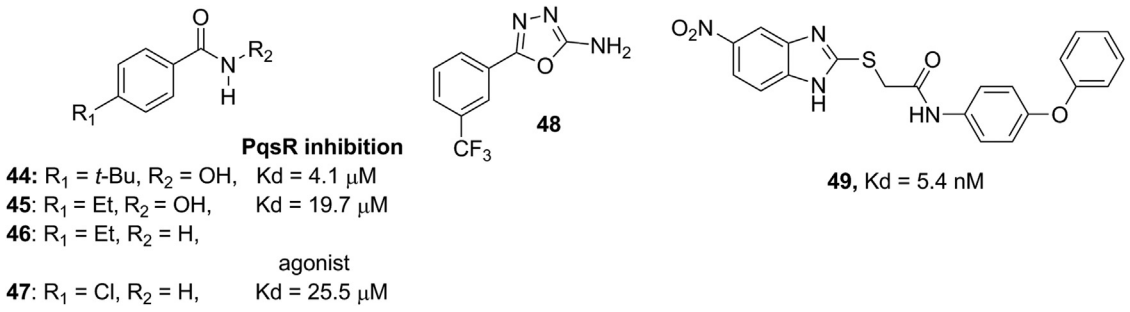

FIGURE 25.7 Structures of selected PQS inhibitors.

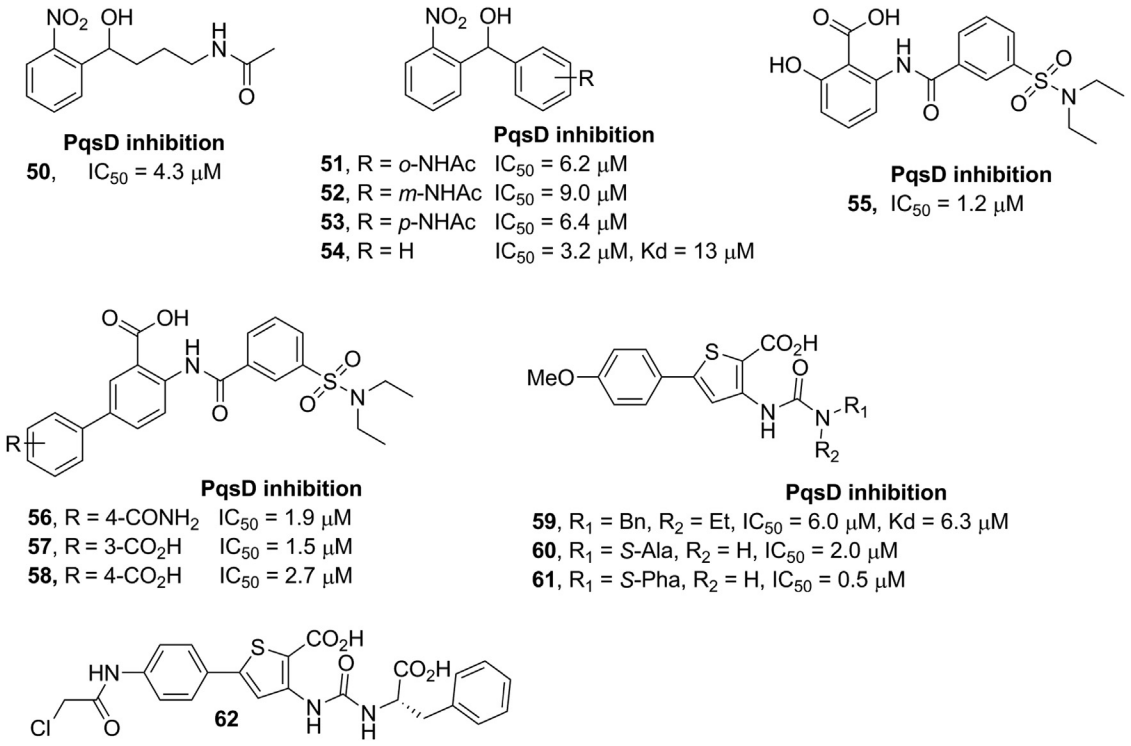

FIGURE 25.8 Structures of PqsD inhibitors.

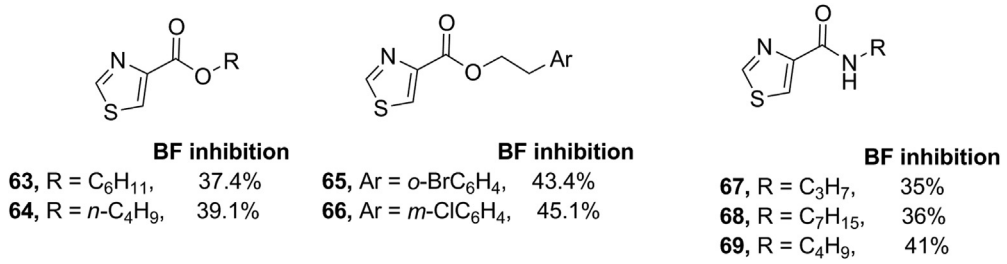

FIGURE 25.9 Structures of IQS inhibitors.

bind in the ACoA channel of PqsD. Instead of competing for the active site of the enzyme, inhibitors prevented approaching of the native ligand to the enzyme.

Additional highly active PqsD inhibitor (**59**; Fig. 25.8) that also binds in the ACoA channel was used for further structure optimization (Sahner et al., 2013). As a result, more active derivatives **60** and **61** were obtained (Fig. 25.8). In the case of these derivatives, stereochemistry proved important (*S* vs *R* configuration on urea part), with *R*-isomers being significantly less active. From derivative **61**, irreversible inhibitor **62** was developed, which retained *S*-Phe part but have *N*-(2-chloro)acetamide on the opposite side, enabling covalent binding to PqsD.

25.4.3.4 Inhibitors of IQS signaling pathway

IQS signal circuit is the last signaling pathway identified in *P. aeruginosa* (Lee & Zhang, 2015). Under low-phosphate conditions it can partially overtake the function of Las pathway. The signaling molecule of IQS pathway, identified as aeruginaldehyde, is a byproduct of pyochelin biosynthesis and is involved in the control of many QS- and virulence-associated genes expression. This makes IQS an attractive target for developing QSI.

A series of thiazole-4-carboxylic acid derivatives were designed as inhibitors of biofilm formation and other virulence factors production (Li et al., 2018). Two series of derivatives, esters and amides, showed moderate effect on biofilm formation under low-phosphate conditions, with phenetyl esters (**65** and **66**; Fig. 25.9) as the most active

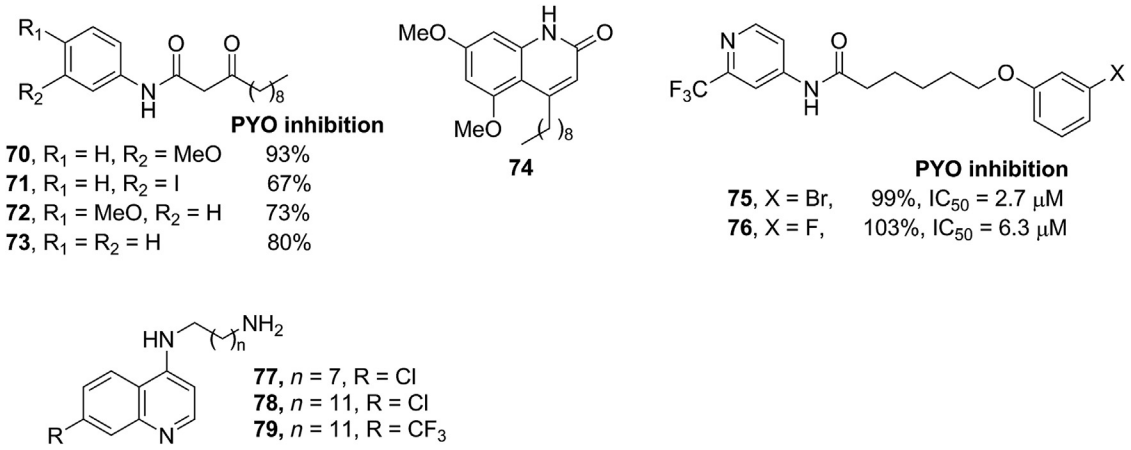

FIGURE 25.10 Structures of pyocyanin inhibitors.

ones. Derivative **66** also inhibited expression of PqsA and RhlA, as well as production of rhamnolipides (43%) and pyocyanin (30%) at 20 μM.

25.4.3.5 Virulence factors inhibitors

Based on structural similarity with 3-oxo-C12-HSL, a series of substituted anilide derivatives that inhibited pyocyanin were synthesized (Hodgkinson et al., 2012; Morkunas et al., 2012) with derivatives **70**−**73** (Fig. 25.10) being most active ones. Derivatives **70** and **72** also inhibited elastase production.

A 2-quinolone derivative **74** (Fig. 25.10) inhibited the production of pyocyanin by 86% at 200 μM. Molecular docking model with LasR indicated that compound **4** may bind to the same site like 3-oxo-C12-HSL, thus Las signaling pathway was suggested as its target (Morkunas et al., 2016).

A series of aminopyridines were developed as pyocyanin inhibitors (Miller et al., 2015). Pyridine ring, length of alkyl chain, and the presence of additional aromatic electron-deficit part on the opposite side were identified as important structural parameters for the observed activity. Derivatives **75** and **76** (Fig. 25.10) showed inhibition of pyocyanin production at low micromolar concentrations. Although derivatives were designed to mimic natural LasR and RhlR ligands, antagonistic effect was not achieved.

Recently, we have reported synthesis of a series of long-chain 4-aminoquinoline derivatives with PQS inhibitory activity (**77**−**79**; Fig. 25.10) (Aleksić et al., 2017). Within this series derivatives **78** and **79** were the most active inhibitors of biofilm formation (with IC_{50} values 63 and 69 μM, respectively) and pyocyanin production (IC_{50} value of 2.5 μM).

Successful maintenance of biofilm requires the presence of various divalent cations, including Fe^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}. Exposure of *P. aeruginosa* to subinhibitory concentration of antibiotic (40 μM) *Nitroxoline* (5-nitro-8-hydroxiquinoline) (Fig. 25.11) inhibited biofilm formation in a concentration-dependent manner (Sobke et al., 2012). Since 8-hydroxiquinolines act as chelators (Rahier, Noiriel, & Abousalham, 2016), it is assumed that chelation of the essential ions in biofilm is the major mechanism of this antibiotic.

Imino derivatives obtained from 2-formyl quinolines were evaluated as inhibitors of biofilm formation and potential antagonists of PqsD (Sangshetti et al., 2015). Two of the derivatives (**80** and **81**) were strong biofilm formation inhibitors with IC_{50} values 12.97 and 15.63 μM, respectively. Importantly, these compounds also showed bactericidal activity at these concentrations. Series of 3-phtalazine-quinolines exhibited moderate inhibitory activity on biofilm formation, but their bactericidal activity was significantly lower (Zaheer, Khan, Sangshetti, Patil, & Lohar, 2016). Derivatives **82**−**84** (Fig. 25.11) were active against biofilm formation and did not affect the growth of planktonic cells.

A series of amide derivatives of 2-amino and 2,3-diamino-4-quinolones exhibited strong inhibition of biofilm formation (Espinosa-Valdés et al., 2019). Biofilm formation inhibitory activity depended on alkyl chain length, with C10−C16 derivatives being the most active ones (Fig. 25.11). Dodecanoil amides, **85** and **89**, also reduced pyocyanin production.

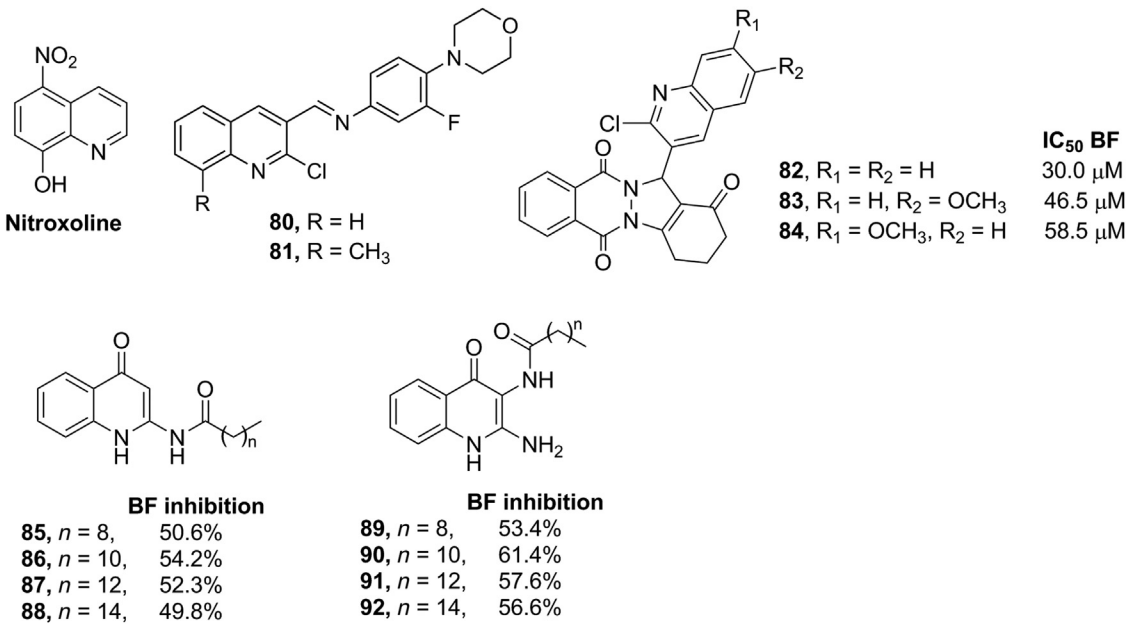

FIGURE 25.11 Structures of biofilm formation inhibitors.

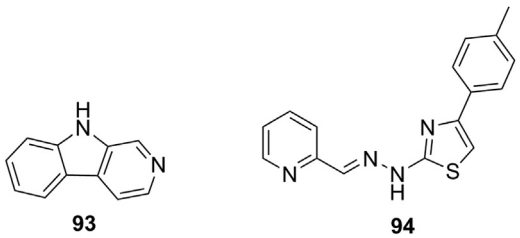

FIGURE 25.12 Structures of ligands **93** and **94**.

25.4.3.6 *Metal complexes as quorum sensing inhibitors*

In contrast to organic compounds, where developing of active substances is based on the structural similarity with natural AIs or modeling with either appropriate QS receptors or enzymes, investigation of metal complexes is based on the data that many of them express antibacterial activity (Al Zoubi, Al-Hamdani, Putu Widiantara, Hamoodah, & Ko, 2017; Ng et al., 2013; Osowole, Ekennia, Olubiyi, & Olagunju, 2017; Saad & Al-hazmi, 2015). An anti-QS activity of five Cu(II) complexes prepared from pyrimidine, pyrazine, quinazoline, and phthalazine was reported (Glišić et al., 2016). All of them inhibited production of AHQ, while stimulating the production of C4-HSL and 3-oxo-C12-HSL. The complex $[Cu(qz)_2(H_2O)_2](NO_2)_2$ with quinazoline showed inhibition of biofilm formation by 60% at 250 μg/mL.

A group of three Cu(I) tetrahedral complexes $[CuL(PPh_3)_2]X$, where $X = Cl^-$, Br^- or I^- and L is 9H-pyrido[3,4-b] indole (**93**; Fig. 25.12) inhibited biofilm formation, elastase, pyocyanin, alginate, and EPS production, as well as swarming motility (Al-Shabib et al., 2019). Those complexes showed a broad-spectrum anti-QS activity, inhibiting QS in different medically relevant pathogens such as *E. coli*, *Chromobacterium violaceum*, *S. marcescens*, *Klebsiella pneumoniae*, and *Listeria monocytogenes*.

Complex Co(III) $[CoL_2]BF_3$ (LH = **94**; Fig. 25.12) inhibited biofilm formation (35%−63% at 6.25−800 μg/mL) and pyocyanin production (90% at 12.5 μg/mL), and suppressed 3-oxo-C12-HSL production up to 41% (Borges, Simões, Todorović, Filipović, & García-Sosa, 2018).

25.5 Perspective of antivirulence therapy

Antivirulence strategy is aimed to target the bacterial virulence machinery required to damage the host and cause the disease. The greatest advantage of antivirulence therapies is that there is weaker evolutionary pressure for the

development of resistance compared to currently used growth inhibition-based therapies. Although it is expected that the resistance is unlikely to emerge, the laboratory results have indicated that resistance to antivirulence agents could occur but at the slower rate than the one observed for antibiotics (Ruer et al., 2015).

The effectiveness of QSIs has been observed only when applied early in bacterial growth, when cell density is low. Thus they should preferentially be applied as prophylactic antivirulence agents rather than used for treatment of established infections. But, when used for the treatment of existing infections QSIs should be applied together with other types of antivirulence agents targeting other systems (such as those that disperse biofilms) or as adjuvants for bactericidal agents such as antibiotics or metal complexes. Therefore the search for novel classes of antibiotics should not be neglected.

Despite the large number of molecules with reported antivirulence activity only a few clinical trials have been conducted due to numerous obstacles that emerge while moving from the laboratory studies to the clinic application. Some of the obstacles include toxicity, lack of stability, efficient delivery, or potency in animal models. Extensive SAR and molecular modeling studies enabled identification of the most promising antivirulence agents with IC_{50} values in nanomolar or submicromolar concentrations, but further structural modifications are expected to help overcome toxicity and stability issues.

To secure efficient delivery of antivirulence agents, nanoparticles (NPs) or different types of biologically inert materials could be used as carriers. Nanocarriers (metallic, organic, carbon nanotubes) could be biologically inactive or may exhibit antimicrobial or antivirulence characteristics by themselves, thus having synergistic activity with the antivirulence agent (Baptista et al., 2018; Lee, Kim, Cho, & Lee, 2014). Combined use of antivirulence agents and NPs could also help to overcome toxicity issues. The potency of antivirulence agents is limited by their low bioavailability due to poor membrane transport. NP carriers can facilitate the drug's entry into the cell by endocytosis or through interactions with surface lipids. Reduced toxicity and prolonged activity could be achieved by slow release of active compound from functionalized materials (Broderick et al., 2014; Kratochvil, Tal-Gan, Yang, Blackwell, & Lynn, 2015).

Despite the described obstacles we feel that antivirulence therapies are a promising approach to fight MDR bacterial pathogens, which are on the verge of becoming untreatable. The diversity of molecular structures found in nature and structure-based rational drug design have enabled the development of numerous molecules with potent QSI activities. All these, together with a growing number of patent applications focusing on QSI approaches, make us optimistic that antivirulence therapy will soon find its way from bench to clinic.

Abbreviations

AHL acyl homoserine lactone
AHQ alkyl hydroxyquinolones
AI autoinducer
AIP autoinducer peptide
AQ alkylquinoline
BF biofilm
DKP diketopiperazine
EO essential oils
EPS exopolysaccharides
IQS integrated quorum sensing system
LPS lipopolysaccharides
MDR multidrug resistance
PYO pyocyanin
PQS *Pseudomonas* quinolone system
QS quorum sensing
QQ quorum quenching
QSI quorum sensing inhibitor
ROS reactive oxygen species
SAM S-adenosylmethionine
T3SS Type III secretion system

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Chapter 26

Biomedicine: biodiversity's panacea? Context of commodification

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26.1 Introduction

Biodiversity has ebbed and flowed over the millennia, its trajectory characterized by meandering changes punctuated with species explosions and massive extinction events. It takes several million years after an extinction event for the extant species to diversify to levels comparable to what existed prior to the extinction event (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). Scientists charge humans with the ongoing sixth global extinction event and caution that if we do not curb our contributions to climate alteration, habitat destruction, exploitation of nature, and dissemination of introduced species, biodiversity loss will continue and may accelerate (Chapin et al., 2000; Díaz, Settele, et al., 2019). Biodiversity loss rates are estimated at 100 times or more what they were prior to humans (Pimm et al., 2014; Lamkin & Miller, 2016). An assessment of global rates of seed plant extinction suggest 2.3 species have gone extinct per year for the past 2.5 centuries, suggesting rates of seed plant species loss at about 500 times the rates that would be expected in the absence of humans (Humphreys, Govaerts, Ficinski, Lughadha, & Vorontsova, 2019).

The idea that commodification of biodiversity for medicinal benefits could incentivize the preservation of biodiversity has been periodically explored (see Hough, 2014 for a brief history). This book documents a resurgence in interest around this concept. The authors demonstrate the broad variety of ecosystems within which biodiversity has been shown to provide medical promise, with examples drawn from plants and the plant microbiome (Chapters 1, 19, 20, 24, 26, 28, and 29), fungi (Chapters 2, 13, 21, 23), animals (Chapter 14: Anticancer activities of marine macroalgae: status and future perspectives), and marine species (Chapter 4: Biomining fungal endophytes from tropical plants and seaweeds for drug discovery, and Chapter 15: Insights into the bioactive compounds of endophytic fungi in mangroves). This book's authors share inspiring data on the use of natural products to address cancer (Chapters 5, 9, 10, 11, and 15), fungal infections (Chapter 8: Human genetic diversity in health and disease), and diabetes (Chapter 13: Mushroom and plant extracts as potential intervention supplements in diabetes management). This book is full of promising success stories related to drug breakthroughs, emerging technologies, and local and social benefits associated with research on and the development of biomedicines from biodiverse areas, but there is little discussion around some of the concerns raised by the commodification of nature or the commodification of human health. While a discussion around the ethics associated with a for-profit medical system is beyond the scope of this chapter, widespread acceptance of this paradigm is driving the commodification and monetization of nature discussed here.

In discussions around development of biomedicines from biodiverse systems, challenges that occur along the entire value chain are seldom adequately explored. If the link between biodiversity and biomedicines is to lead to sustainable management of nature and future drug development, all points along the value chain linking the environmental and

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health sectors will need to be thoroughly addressed. The fields of taxonomy, systematics, and ecology have a vast amount of work left to adequately classify and catalogue species diversities (including multispecies "organisms," such as microbiomes, symbionts, endophytic fungi, etc.) and understand habitat function and needs in order to enable humans to manage these systems. Anthropologists, historians, ethnobiologists, and social scientists have significant work to do documenting the composition and preparation of traditional medicinal products. Geneticists, cell biologists, and chemists face challenges in exploring the medicinal potentials of biological materials. Conservation biologists strive to demonstrate which conservation approaches are the most effective at maintaining the highest amounts of biodiversity and/or preserving enigmatic species, while nation-states, drug companies, and traders of medicinal products seek to develop sustainable supply chains and markets.

As with all solutions to complex challenges, there are pitfalls and opportunities associated with promoting the idea of preserving biodiversity by exploiting it for medical benefits. Ideally, conservation biologists, drug companies, policy makers, planners, and local communities will continue to collaborate to understand the ecological, economic, and social incentives and constraints that will lead to sustainable exploration and use of products developed from nature.

26.2 A brief primer on the concept of ecosystem services as related to biodiversity and biomedicine

An in-depth study on the anthropogenic disruption of ecological systems led to the development of the concept of "environmental services" (Wilson & Matthews, 1970). Soon reminted as "ecosystem services," the basic idea is that nature supplies humans with a variety of undervalued services that provide us with basic welfare, stability, resilience, health, and quality of life. While there are two main pillars of thought in the ecosystem service concept (i.e., the conservation biology approach and the environmental economics approach), it is widely accepted that humanity's relationship with nature can be understood and improved via economic valuation. The commodification of biodiversity is a potentially dangerous outcome of increasingly widespread acceptance of the assumptions and relationships implicit in the ecosystem services concept (Kosoy & Corbera, 2010). Many natural scientists and economists continue to refine the concept of "ecosystem services" (Bennett et al., 2015; Costanza et al., 1997; Díaz, Pascual, et al., 2019; Ehrlich & Mooney, 1983; Fisher, Turner, & Moring, 2009; Gómez-Baggethun, de Groot, Lomas, & Montes, 2010; MEA, 2005), while others criticize the shortcomings of the approach and propose new paradigms of thought (Lele, Springate-Baginski, Lakerveld, Deb, & Dash, 2013; Silvertown, 2015). The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES)-supported "Nature's Contributions to People" (NCP) concept reiterates the ecosystem services concept with an elevated consideration of indigenous and local knowledge and culture's role in defining humanity's relationship with nature (Díaz, Pascual, et al., 2019), though some argue the ecosystem services concept already accommodates these concerns (Braat, 2018).

Nature is increasingly being plundered for the benefits it provides to human populations, including the physical and biological services on which we depend. Lele et al. (2013) argue that the scientific community has confounded the concept of biodiversity as something independent of ecosystem services, leading to a misassessment of the value of biodiversity, which they argue has no intrinsic value. The biomedicine to biodiversity concept provides a framework for valuing biodiversity for medical benefits, rather than an intrinsic value. Maintenance of more biologically diverse systems increases the likelihood of the existence of medicinally useful species or natural products (Hough, 2014). It is the hope of groups like the "Biodiversity to Biomedicine Consortium" (Neergheen-Bhujun et al., 2017) that linking ecosystem and human health will improve human understanding and appreciation of our connection to natural systems. Because of our inherent self-interest, an increased appreciation of our dependence on ecosystems and our cultural relationships with them would ostensibly lead to increased conservation efforts and preservation of biodiversity and indigenous practices associated with them. In other words, the idea of using an ecosystem services-based argument to protect biodiversity for biomedicine relies on human self-interest: the hope is that humans care about our own health and well-being enough that once we realize the potential benefits we stand to gain from protecting biologically diverse systems, we will take action.

Although physical goods (e.g., timber) harvested from ecosystems are assigned prices based on economic systems, many of the provisional and not-material services ecosystems provide are cascading and much more difficult to quantify and economically value. For example, how much is a mangrove swamp worth that prevents massive flooding (property damage) and therefore also prevents problems associated with flooding (infrastructure damage, personal property damage, drinking water contamination, crop failures, malaria outbreaks, civil unrest, etc.)? An economic metric related to impacts of flood risk on property values or insurance premiums may be developed rather easily. It is more challenging

to assign a value to the benefits provided by a healthy shoreline, including stable housing, disease reduction, healthy seafood populations, and recreational opportunities because of habitat and beaches maintained by the stabilizing mangroves. It is even more difficult to calculate the benefits that ecosystems provide in terms of social and community security and stability. Sometimes, putting a value on knowledge we generate and physical and psychological experience we gain from nature is absurd.

The potential biomedical benefits that biodiverse systems could supply are comparably difficult to quantify. Biodiversity has evolved over millennia, forced by environmental pressures and interactions, chance, and happenstance. The calculations are difficult because the benefits being enjoyed are taken for granted and are often only recognized as benefits once the ecosystem providing them is lost. Further, the economic systems that are well established around the globe tend to look at the benefits ecosystems provide as "free" (de Groot, 1987). The ecosystem services concept arose as an attempt to correct this notion. It was thought that by monetizing ecosystem services, they would be valued and conserved accordingly. The more recent Nature's Contributions to People (NCP) seeks to broaden the impact the ecosystem services concept sought to achieve, particularly in relation to understanding biodiversity within an indigenous and cultural context (Díaz, Pascual, et al., 2019).

26.3 Biodiversity, bioprospecting, and human welfare

26.3.1 Biodiversity

Biodiversity may seem like a straightforward concept, but upon closer examination it becomes apparent that there are many ways the word can be interpreted. The agreed upon definition of biodiversity will determine metrics of successful conservation. The word "biodiversity" can refer to diversity of species, diversity of populations or other taxa, or even diversity of ecological processes (Myers et al., 2000). Diversity of one taxon may or may not be related to diversity of another in the same geographical space, so an area rich in plant species may or may not be rich in frog species (Pimm et al., 2014). In Europe there is only a 53% overlap between the areas that support significant plant diversity and those that support significant bird diversity (Willis, 2017).

As genetic and molecular biology techniques continue to develop, definitions of biodiversity are increasingly inclusive of inter- and intraspecific genetic and chemical diversities. Highly diverse areas have been dubbed "hotspots"; these may be rich in species, taxa, or ecological processes, and/or have a high diversity of endemic, unusual, or rare species that are irreplaceable (Myers et al., 2000). In 2000 scientists estimated that roughly 44% of vascular plant species and 35% of vertebrate species were found only in concentrated so-called hotspots that comprised less than 2% of the Earth's terrestrial surfaces; previously this diversity existed on 12% of the Earth's land surfaces (Myers et al., 2000). Areas of the globe rich in plant species show accelerated rates of extinction (Humphreys et al., 2019). Biodiversity hotspots are also mapped on the basis of vulnerability; therefore, the fate of biodiversity hotspots will drive the rates of biodiversity loss. Yet, some of the highest distributions of medicinal plant species occur outside the formally designated biodiversity hotspots (Díaz, Settele, et al., 2019).

Most biodiversity hotspots occur in developing countries, where local populations tend to have limited technological and economic capacities to protect biodiversity resources and a significant need both for biomedicinal resources and for the potential revenue they could generate (WHO, 2002). Natural products can provide important healthcare in subsistence communities in the biodiversity hotspots and elsewhere. They may also form the economic base, such as in parts of the Himalayas, where the collection and sale of the Caterpillar fungus (*Ophiocordyceps sinensis* (Berk.) G. H. Dung, J.M. Sung, Hywel-Jones & Sparafora) may represent up to 90% of household incomes (Winkler, 2008). Harvest of medicinal species like the caterpillar fungus may support these communities now, but threats from overharvesting, unsustainable harvesting, habitat destruction, and other anthropogenic pressures imperils the sustainability of wild-harvesting (Shrestha & Bawa, 2013). The same pressures are active around the world in different intensities. Bussmann, Sharon, Vandebroek, Jones, and Revenue (2007) note that the most pressing problems facing medicinal plant species of the Andes was habitat destruction due to urban expansion, agricultural pressures, deforestation, natural resource extraction, and climate change. The unsustainable harvest of rhinoceros horn for use in traditional medicines provides an illuminative example of how market demand can drive humans to kill endangered species for profit, disregarding deleterious consequences on the future of the species, the sustainability of their profit-source, or the local ecosystems.

The meaning of "biodiversity" can also vary by spatial scale. Across the landscape, biodiversity can be described across spatial scales as "alpha," beta," and "gamma" diversities (Jost, 2007; Whittaker, 1960). Alpha diversity is local diversity (e.g., within a valley in Punjab). Alpha diversity could also be used to describe the diversity of a hotspot. Gamma diversity represents regional diversity (e.g., the state of Punjab or the Indian subcontinent). A region could

have relatively high gamma diversity even with relatively homogenous set of species over the majority of the area and one or two hotspots of diversity. Beta diversity describes the ratio between alpha and beta diversities, so essentially describes how many instances of *different* high alpha diversity occur across a region.

Understanding biodiversity at a landscape scale is important because it helps to guide conservation priorities, policies, and metrics of success. Agricultural conversion helps illustrate potential outcomes associated with identification of medically desirable species and subsequent cultivation thereof. In terms of plant species diversity, shifting large portions of Central American forest to shade-grown coffee production (diverse overstory tree species, but coffee-dominated understory) may increase the alpha diversity relative to monocultural crop production but decreases the beta and gamma diversities relative to what could be maintained with a patchwork of monocultural cropping and native forest remnants. Intensive "wild-cultivation" of medicinal species could similarly lead to an overall decrease in regional biodiversity.

26.3.2 Biomedicine and bioprospecting

The natural world is bountiful with medicinal potential. For thousands of years cultures around the planet have codeveloped systems of medicines with the ecosystems surrounding them (Cragg & Newman, 2013). According to the World Health Organization (WHO), traditional medicine (using natural products) is the primary source of medicine or provides important complementary medicinal benefits to most of the world's population (WHO, 2014). Traditional Chinese Medicine (TCM), derived primarily from nature-based sources, will be officially integrated into the Chinese mainstream healthcare system by 2020 (Willis, 2017). Use of herbal and other natural preparations is not limited to developing nations; most Germans, for example, use herbal medications (Willis, 2017).

Documented global integration of nature-based pharmacopeia began at least 1200 years ago, when Arab scholars chronicled and integrated medicinal systems developed by Mesopotamians, Greco-Romans, and Indians (USNLM, n. d.). Thereafter international trade allowed for medical practitioners to prescribe local and international medicines, sometimes in complicated formulations. As globalization continues today, many indigenous systems of medicine continue to incorporate local and traditional knowledge into their approach, supplementing their pharmacopoeias with biotic materials from other parts of the world (Bussmann et al., 2007). Over 28,100 plants have documented medicinal uses, but less than 16% of these are cited in medicinal regulatory publications (Willis, 2017).

While justified concerns are raised over the loss of traditional knowledge systems related to natural medicines, it should be remembered that not all traditional knowledge is grounded in measurable science. For example, in various African countries albino humans are hunted for their body parts, considered valuable in traditional medicines (McNeil, 2009).

Direct trade in medicinal plants and animals and their raw derivatives is worth billions of dollars. Drugs developed from medicinal species particularly bacteria and fungus offer even more lucrative scenarios to pharmaceutical developers. Periodically pharmaceutical companies have been lured by the lucrative promises of new drugs held by areas rich in biodiversity (Leonti, 2015). As of 2008, 80% of drugs in use in the United States were based on natural sources (Roberson, 2008). At the same time, it was estimated that over 15,000 promising species were threatened or endangered and biodiversity loss was costing humans at least one major new drug discovery every 2 years. The pace of biodiversity and biomedicinal losses has only increased over the past decade.

Evolution has refined biologically active secondary metabolites through a long history of natural selection or mutation impossible to duplicate in a laboratory. Evolutionary processes have led not only to complex and novel structural configurations of molecules, but also to highly specific, biologically-functional molecules (Cragg & Newman, 2013; Newman & Cragg, 2012). However, evolutionary processes that led to the development of genetic diversity and diverse secondary metabolites of living organisms were not doing so with the purpose of developing biomedicine. It is incumbent upon human scientists and researchers to determine how these compounds may inform our approach to healthcare moving forward.

All organisms living on this planet depend on other organisms for their survival. Sponges are a symbiotic community of animals, fungi, and bacteria. Together these three organisms produce bioactive secondary metabolites and compounds, antibiotics, and cytotoxic proteins that protect their community directly and indirectly. Müller et al. (2004) provide an excellent summary of medicinal potentials of some of these compounds. All assayed plant species have symbiotic relationships with one or more endophytes, microorganisms that live on or in plant tissues (Strobel & Daisy, 2003). Endophytes have been shown to produce antibiotics, antiviral compounds, anticancer compounds, immunosuppressive compounds, and other bioreactive compounds with great potential in improving human health through biomedicine development.

Bioprospecting was promoted as a potential win−win solution that would further the human pharmacopeia while also conserving biodiversity and ensuring equitable distribution of profits to shareholders who live in the biodiverse regions of the globe (Rosenthal, n.d.; Roberson, 2008). The term "bioprospecting" emerged in the 1990s and is defined by the Oxford English Dictionary as, "the search for plant and animal species from which medicinal drugs and other commercially valuable compounds can be obtained." Pressures the human population places on nature to access this form of healthcare continue to grow. The WHO guiding document focuses on ways governments can ensure the safety of healthcare and maximize its potential, while paying little attention to the impacts of exploiting nature for medicines. The World Health Organization's 2005 Human Health report (Corvalan, Hales, & McMichael, 2005), developed out of the Millennium Ecosystem Assessment contains the word "medicine" only twice. Similarly, the paper that delineated the IPBES conceptual framework contained "medicine" only once and suggested that the medicinal value that nature provides is a benefit that nature provides with negligible anthropogenic inputs (Díaz et al., 2015).

If care is not taken, the important resources that nature provides or that human communities coproduce in terms of traditional and complementary medicines will not be used sustainably and will eventually cease to exist. The same threat exists from mismanaged bioprospecting by companies, governments, and individuals hoping to isolate bioactive compounds and build pharmacopoeias of new drugs. The term "biopiracy" soon emerged to describe a fast-emerging problem, "the practice of commercially exploiting naturally occurring biochemical or genetic material, especially by obtaining patents that restrict its future use, while failing to pay fair compensation to the community from which it originates" (Biopiracy, n.d.). Advances in molecular and cellular biology increasingly provide new opportunities for exploration of genetic and metabolic diversities in species and systems, while two Nobel prizes awarded for drugs developed from natural products have reinvigorated the quest for natural product discovery (Shen, 2015). Progress in molecular and cell biology has enabled exploitation of secondary metabolites and biomaterials (Müller et al., 2004). These advances and their ramifications for bioprospecting and biomedicine development are detailed in other chapters of this book and will not be discussed in detail here.

Trade in medicinal species and drugs developed from nature are both increasingly lucrative. Of 1073 new chemical entities (NCEs) developed between 1981 and 2010, nearly 70% of the antifungal, antibacterial, antiviral, and antiparasitic agents and 75% of anticancer agents were derived or inspired by molecules found in nature (Newman & Cragg, 2012). Roberson (2008) estimated that in 1995 the average new plant derived drug was worth over US$90 million to pharmaceutical companies and provided nearly US$450 million in benefits to society; she noted in the mid-1990s global trade in medicinal plants was worth more than US$24 billion, with plant-based drugs worth more than US$84 billion. By 2000 US consumers spent more than $17 billion on herbal medicines and the global herbal medicine market was estimated at over $60 billion (Willis, 2017). In 2006 one quarter to one half of the US$640 billion pharmaceutical industry's products were derived from genetic resources (i.e., nature); drugs and therapies developed from traditional remedies in 2006 were worth US$32 billion per year (Richerzhagen, 2011). By 2012 trade in Traditional Chinese Medicine alone was worth US$83 billion (Willis, 2017).

26.3.3 Human welfare and ownership of nature

The "tragedy of the commons" occurs when a resource or benefit exists in the public sphere and people exploit it for personal gain rather than conserving it for the benefit of themselves and others (Hardin, 1968). Some argue that biodiversity is a public good (Richerzhagen, 2011), while others suggest that treating a resource as a public good ensures its destruction (Smith, 1981). While it may seem logical that we could place biodiversity into the stewardship of humanity and we would all be committed to protecting the environment for the medicinal secrets yet unlocked (i.e., for our own future benefits), Hardin argues that in a stable society rational people will exploit the commons for shortsighted, personal gain. Paradoxically, what appear to be individually rational strategies provide irrational outcomes for the community (Ostrom, 1990). Ostrom (1990) suggests there are three avenues for protecting natural resources such as biodiversity: central or federal regulation, local or stakeholder regulation, and privatization. While some examples of community ownership show promise at conservation, if not carefully managed perpetuating the idea of "ownership" may perpetuate the commodification of an unmeasurable resource and fuel broader divisions of wealth.

Discussing how to best share biodiversity's benefits commodifies nature and inclines us to think of biodiversity in terms of property (Hamilton, 2006). In particular, perceiving genetic resources as "sovereign resources" over which communities or nation-states should hold power and ownership establishes them as property and moves us away from our thinking of nature as "common heritage" shared by humanity. Ironically, this idea of ownership is sometimes promoted by people interested in providing local stakeholders with more standing and leverage over the ecosystems in

which they live, but the idea opens the door to the monetization of their environment, which leads to pressures by companies for development and ownership.

The revenue streams created in biomedicines are unequally distributed among stakeholders in the product streams. As an example, in Pakistan the nuts from the Chilgoza pine (*Pinus gerardiana* Wall.) are considered medicinal (Aziz et al., 2018). Local communities derive income from harvesting and selling the nuts but are not involved in the value-added steps of roasting and packaging the nuts for sales and therefore do not enjoy most of the profits from the medicinal plants they are harvesting.

Human history is riddled with stories of controversy over "ownership" of various species. Agricultural history provides many examples of species over which wars were fought and fortunes were built, such as coffee, chocolate, and tea. In many instances the regions or communities from which these products emerged have not enjoyed the profits. Coffee, for example, is native to Ethiopia, where the promotion of shade-grown coffee products as environmentally responsible has helped to curb deforestation and provide local communities with continued "forest benefits" (Tadesse, Zavaleta, Shennan, & FitzSimmons, 2014). In other regions of the globe, where coffee is not native, growing coffee with diverse overstory trees provides some species refugia, but is not a substitute for preservation of remaining forest remnants (Philpott et al., 2008). In no case does Ethiopia directly benefit from coffee production in other parts of the world.

26.3.4 Producing medicines from nature

Consumer willingness to pay high prices for natural products can lead to cultivation of the plants, fungi, animals, and algae used to make these products. However, intensive cultivation or breeding programs may not meet consumer demands and may therefore not prevent overharvesting (Cunningham & Long, 2019). When prices fall, farmers may convert their production systems to more lucrative crops, leaving traders to rely on wild-harvest products even when they are unsustainably harvested. The continued decline in wild populations of *Swertia chirayita* (Roxb. ex Fleming) H. Karst. across India, even in light of a federal prohibition on most wild-harvest and trade, demonstrates the lack of efficacy that federal prohibitions can have if they are not backed by strong enforcement (Cunningham, Brinckmann, Schippmann, & Pykurel, 2018).

Biota and its derivatives have been traded nationally and internationally for millennia. Recent advances in genomics, metagenomics, microbial cultivation, and synthetic biology promise a renaissance of engineered or synthetic products inspired by natural products (Shen, 2015). However, wild products are often considered to have more value by consumers, even when there are no or nominal discernable difference in medicinal properties between wild, cultivated, or synthetic products. Producing (or at least marking products as) medicines from wild products has a higher economic return than producing medicines from cultivated or synthetic compounds (Müller et al., 2004).

Overharvesting of wild populations can be deleterious to species' genetic diversity. Although it is tempting to think of a species as a particular set of genes, one must remember that every individual in that species has a unique genetic makeup. If only a small percent of a population makes particular alkaloids, maintaining the species while losing that population means a loss for biomedicine development. Wild-harvest can have significant impacts on the future of medicinal species and the ecosystems of which they are a part. In Pakistan, demand for the nut of a medicinal pine tree has led to overharvesting, which significantly threatens establishment of new trees (Aziz et al., 2018).

In addition to overharvesting and destruction of natural ecosystems, consumer or corporate demand for natural products can lead to adulteration of natural products and substitution of alternative products without the same medicinal properties (Cunningham et al., 2018; Cunningham & Long, 2019). This may mislead drug development and may impact conservation of nontarget species.

Many people see little conflict in the ethics of raising plants, fungi, and algae for harvest, but animals can pose significant moral and ethical dilemmas. For example, lions (*Panthera leo* Linnaeus) are "farmed" for various reasons, including for inclusion of their bones in traditional medicines across Southeast Asia and Africa (Coals et al., 2019). Lions are listed in CITES Appendix II, signifying they are a species in which trade must be controlled in order to ensure sustainability. CITES requires that nations exporting species on this list set quotas on the number of bodies that can be traded. While farming of endangered species is well established, it remains to be seen whether the practice will spread and continue to be supported.

26.4 Next steps

26.4.1 Developing a better understanding of biodiversity and conservation

Developing local, national, and international policy-structure and enforcement bodies around the conservation of biodiversity would be a formidable challenge in and of itself. Adding complexity to the process is the fact that our current

knowledge of local and global biodiversity is limited, as is our understanding of how to best conserve biodiversity and support the processes that maintain it. Additionally, cultural histories, conflicts, economic growth, geopolitical relationships, and climate change can all influence how communities and nations interface with the natural environment. Mismanagement of conservation-related conflicts can have negative effects on biodiversity, human health and well-being, and human livelihoods, which can in turn impact human decisions around biodiversity conservation.

26.4.1.1 Documentation of biodiversity and biomedical implications

In the context of this book the documentation of biodiversity is a multistep, iterative process. The first is a daunting task in and of itself, which is developing catalogues of species and monitoring their interrelationships in the ecosystem. As many as 80% of living organisms on the planet are yet to be discovered by scientists; biodiversity research requires more field work, more exploration, and more interfacing with indigenous communities (Wilson, 2017). In particular, invertebrate species are largely undocumented and may be central to the survival of many vertebrate and plant species, plus microbial species associated with their microbiomes (Régnier et al., 2015). Scientists need to document biologically active metabolites and other biological products of organisms and microbiomes. Finally, scientists will need to assess the potential therapeutic applications and biomedicine development from these species and systems. In order for this value-chain of knowledge and product development to remain sustainable, much attention will need to be given to cross-disciplinary communication and support. Significantly, these steps and the follow-on steps of drug development and testing will take significant amounts of time and require ongoing financial support. Newman (2016) estimates decadal to multidecadal time frames are required to develop drugs from natural products.

The scientific community notes the current state of the world's biodiversity is grossly underdocumented and hardly understood (Pimm et al., 2014). Similarly, the potential for medicinal-product development from each species is undocumented. In their 2013 review, Cragg and Newman found the current literature reported only up to 6% of the world's estimated 300,000−500,000 vascular plants have been investigated pharmacologically and 15% have been investigated phytochemically. There are a wealth of unexplored organisms and processes that hold medical potential. One problem is that traditional scientific literature links specific species with specific benefits and rarely discusses the role of biodiversity in preserving the species and the benefit (Hough, 2014). Biologically and chemically active compounds that vary by developmental stage or across plant tissue type have been hardly considered by science. Few studies exist on the biological potential for medicine development in marine systems. Less than 1% of observed microorganisms were isolated and cultivated by 2013, representing another hardly explored drug-discovery resource (Cragg & Newman, 2013). Massive local and international efforts have been initiated to build databases of species diversity and distributions.

26.4.1.2 Assessment and documentation of conservation outcomes

Continued international efforts to document biodiversity and species ranges are essential, as are efforts to better understand the impacts of ongoing conservation efforts and biotic and abiotic pressures on ecosystem dynamics and structures. "Our incomplete taxonomic knowledge impedes our attempts to protect biodiversity. A renaissance in the classification of species and their interactions is needed to guide conservation prioritization" says esteemed ecologist E. O. Wilson in a recent publication (Wilson, 2017). Although biodiversity loss is a well-established phenomenon (WHO, 2019), the solutions are much less clear. Largely, documentation of sustainable conservation solutions to preserve biodiversity are lacking (Redpath et al., 2013). Once underway such documentation will empower scientists to provide policy makers with better assessments of potential conservation outcomes. More specifically, solution-makers not only lack a catalogue of proven, effective conservation practices, but also lack a proven social−political−economic process for development of these solutions.

In 1983 CITES determined that traded and potentially traded species should be monitored. CITES states, "Whenever a Scientific Authority determines that the export of specimens of any such species should be limited in order to maintain that species throughout its range at a level consistent with its role in the ecosystem in which it occurs and well above the level at which that species might be eligible for inclusion in Appendix I, the Scientific Authority shall advise the appropriate Management Authority of suitable measures to be taken to limit the grant of export permits for specimens of that species" (CITES, Article IV, 1983). Appendix I is a list of species threatened with extinction due to trade. While aspirational, the CITES mandate had multiple problems. First, it assumes critically endangered species have been identified and that trade is the only pressure they are facing (i.e., if trade pressures are eliminated the species will be maintained). Second, CITES presupposes documented understandings of species ranges and regional and local capacity to monitor species and their ranges. Third, it assumes effective cross-walking between scientists and trade

policy development. Fourth, it does not address illegal trade. Fifth, it does not accept what Silvertown (2015) notes, which is that imminent extinction creates intense market demand.

Enigmatic species used in traditional medicines may provide excellent case studies to enable the development of better conservation for all species and ecosystems. Some of the world's megafauna and extremely charismatic species are threatened by demand for their body parts to be included in traditional medicines. Rhinoceros horn, for example, has historical and contemporary significance in traditional medicines even though there is no scientifically documented benefit to inclusion of horn-derived-keratin in traditional medicines. Still, cultural drivers support a strong illicit global trade in rhino horn. The conservation strategies used in attempts to preserve the species and curb the illicit trade could provide guidance for how to develop better conservation outcomes for other species (Bending, 2018).

It is not only the impacts and outcomes of conservation practices and programs on individual species or systems that are largely undocumented in the scientific literature. Although ecosystem services continue to be monetized, there are few documented successes associated with various market solutions and conservation efficacy (Richerzhagen, 2011; Silvertown, 2015; see the case studies from the Economics of Ecosystems and Biodiversity project: http://www.teebweb.org/resources/case-studies/). The Conservation on Biological Diversity's (CBGD) promotion of an access and benefits-sharing, market-based approach to ecosystem management, has led to adoption of such in more than 40 countries, but produced few documented successes in terms of decreasing biodiversity losses (Richerzhagen, 2011).

The relationships between conservation, biomedicine, and biodiversity may prove even more complex. Recent research in the cell biology of corals shows that scientific understanding of biological mechanisms that make coral a potentially valuable medical resource may also be applied to coral conservation efforts (Traylor-Knowles & Palumbi, 2014). In other words, diverse ecosystems may hold biological promise in terms of developing solutions to their own decline.

26.4.2 Conflict resolution, cross-cultural communication, and education

26.4.2.1 Guidelines for conflict management in conservation of biodiversity

As humans continue to place pressure on each other and nature, conflicts regarding how to best manage nature will continue to emerge. Discussion around human relationships with nature, including our responsibilities to and interdependencies with biodiversity, can raise complicated ethical conundrums, especially in the contexts of biomedicines. In considerations around the conservation of biodiversity, scientists, business, and local stakeholders, plus governments, may be the primary participants. However, as the issues surrounding conservation of biodiversity and scientific explorations of genetic resources in biodiverse regions continue to scale up, it is increasingly important that these conversations become more inclusive and draw on the insights provided by social scientists, political scientists, and ethicists. Only through increased and respectful interactions will social, natural, and political scientists develop better means of communicating the concerns of their particular specialties in the conversation around interrelationships towards sustainable management of nature (Bennett et al., 2015; Redpath et al., 2013). It is also essential that we continue to learn from past failures and successes in order to determine the best steps forward for the sustainable management of genetic resources and equitable distribution of human welfare. "Getting the institutions right is a difficult, time-consuming, and conflict-invoking process" (Ostrom, 1990, p. 28).

Ethicists and social scientists should be included in the development of biodiversity conservation policy. Coals et al. (2019) utilize an example of trade in lion parts to examine how arguments may be constructed around conservation and competing human interests. They note that arguments can be developed with biases towards conservation, economics, or intrinsic values. In short, they assert that ethically informed adaptive management decisions should include consideration of ecological and environmental science, social science, and formal analyses of ethical arguments (Coals et al., 2019). Analyses of this sort will not provide "the answer," but should provide scientists with a better understanding of the state of the science in terms of how it informs decision-making. Further it should provide policy makers with a better understanding of the potential ramifications of their decision-making.

As trade in medicinal species continues to expand in the global marketplace, there are increasing pressures for scientists and policy makers to better understand and address cross-cultural concerns. Educating people on their connectivity to nature in their local environment can be challenging. Getting people to value ecosystems with which they will never have direct interaction is an extremely daunting task. Scientists face an uphill challenge and educative, respectful, culturally aware communication will be essential to conservation and biomedicine's future.

The Western biomedical–pharmaceutical approach tends to further the disconnect people feel between their own health system and nature. There is a presumed disconnect in the minds of many people between "real medicine" and

"traditional medicine." It is easy to think of modern drugs as "from science" and traditional "herbal remedies" as "from nature." Although some may realize *Penicillium* and other antibiotics were derived from microbes, few consumers think of willow trees, poppies, and poisonous frogs when they take pain killers; *Cinchona* trees and *Artemisia* herbs when they take antimalarial drugs; Gila monsters when they treat their diabetes; or yew trees, sea sponges, and marine snail venom when they take anticancer drugs. Purchasing a medicine that includes turtle oils or powdered pangolin or rhinoceros horn disconnects the consumer of the product from the organism from which the product was derived. Indeed, "there is a significant disconnect at the core of the global environmental crisis—that health policy makers (and the public by and large) do not understand that health outcomes are ultimately dependent on other species and on the integrity of the planet's ecosystems" (Hough, 2014, p. 268).

Some discount the future, contending that human welfare is largely supported by the sacrifice of biodiversity. In fact, researchers note science does not empirically demonstrate that intact biodiversity benefits human welfare and if anything, the reverse is true: in the short-term, humans benefit more from the conversion of biodiverse systems to anthropocentric land-uses (Huynen, Martens, & De Groot, 2004; Lele et al., 2013). It is precisely this lack of holistic, long-term consideration for *sustained* human welfare and technological advances that could enable us to tap into additional benefits of biodiversity that threatens the planet's remaining biodiversity and imperils generations of future medical discoveries. The guiding principle behind the conservation of biodiverse areas for potential drug discovery is that these areas must be preserved as relatively untouched in order for the complex interspecies relationships that have developed over millennia to continue to interact and drive genetic diversification. In other words, these set-aside areas are not "unused" when they are committed to conservation for sustainable drug discovery and development. However, in many instances, it is an armchair luxury scientists enjoy when we proffer advice on how ecosystems should be managed, as most of us are not living in close proximity to these regions and subject to the economic and ecological hardships some communities experience in biodiverse regions.

26.4.3 Policies and regulatory frameworks, plus enforcement

The idea of better communicating how preservation of biodiversity is in human self-interest may empower more lasting conservation solutions, as researchers suggest that effective conservation efforts resonate with social context (Redpath et al., 2013). However, the challenge may be larger than developing better educational frameworks and communication systems. Conservation science is incomplete. There is substantial wealth associated with drug development. If the concept of biomedical potential becomes the driving factor behind the preservation of biodiversity, health-related-profits will likely play a large role in determining the lens through which biodiversity is valued and protected. There are also issues of politicization and outright corruption around quota setting (Coals et al., 2019).

Providing subsidies or incentives to control the economics around exploitation of biodiversity may help to encourage sustainable resource management. However, payments to property owners may perpetuate inequalities rather than supporting sustainable development. Research on Traditional Chinese Medicine (TCM) and Ayurveda can help scientists and decision-makers better understand economic drivers around natural products. Pricing of natural products used in TCM can be related both to resource scarcity and to the viability of current harvest/exploitation (Cunningham & Long, 2019). When consumers are willing to pay high prices for natural products, there are higher incentives for traders and manufacturers to adulterate their products. There are also higher incentives to drive species to extinction (Silvertown, 2015).

As the legal trade in the caterpillar fungus has demonstrated, recognizing the value of a natural product may generate significant income for local populations and government revenues, but it may also endanger the very source of this income (Winkler, 2008; Shrestha & Bawa, 2013). Similar overharvesting concerns have been raised around Peruvian medicinal plants, which are traded on the global market (Bussman et al., 2007). Traditional approaches to increasing socioeconomic security have often been linked to biodiversity loss. Human welfare, health, and well-being tend to be supported by an exploitive, unsustainable relationship with nature (Huynen et al., 2004; Hough, 2014). Additionally, while some have found socioeconomic development and biodiversity loss are positively related, the two are not always spatially coupled; in other words, a local forest may be lost without the local population enjoying any improvement to their health and well-being (Huynen et al., 2004).

Solutions may be both top-down and bottom-up. In efforts to conserve panda habitat outside of designated protection areas in China, researchers developed an inclusive project that incorporated inputs from Chinese villagers, international trade representatives, the Chinese federal government, NGOs, scientists, and companies (Brinckmann et al., 2018). Research found strong support for such inclusive dialogue and promising outcome in terms of the creation of sustainable harvest and trading systems for an endemic medicinal plant species in rural China. However, the driver of their

relationship was based on panda bear habitat preservation. Panda bears are of significant cultural significance in the region; it may be difficult to repeat their successes in other regions without identifying emblematic species associated with other threatened ecosystems.

26.4.3.1 Established institutions and efforts

Well-meaning international bodies continue to develop frameworks for how to best manage dwindling natural resources, including how to preserve biodiverse regions of the globe for biomedical gains. Scientists hoping to move forward these efforts may be well served by considering ongoing efforts and seeking synergies, rather than reinventing a redundant structure. Here we detail a few past and ongoing efforts in these regards.

The United Nations supported the development of the Convention of Biodiversity, conceived of in the late 1980s and inaugurated in 1993, with the stated objectives of promoting sustainable use of biodiversity, conserving biodiversity, and providing for benefits sharing (CBD, n.d.). Established in 1992, the International Cooperative Biodiversity Groups (ICBG) Program was developed with the same goal as the nascent Biodiversity to Biomedicine Consortium (BtBC) (Rosenthal, n.d.; Neergheen-Bhujun et al., 2017), to incentivize conservation of biodiversity via the promise of drug discovery. Both groups are further committed to addressing disparities between developed and developing countries in terms of the costs and benefits of exploring biodiversity for biomedicine. Both groups encourage sustainable development of medicinal resources and equitable sharing of profits from such development, but while the ICBG program is US based (National Institute of Health, National Science Foundation and USAID), the BtBC is an independent, nonprofit global organization with limited connections to federal governments. Prior to the initiation of the ICBG Program, there was little extant scientific work done on best practices for developing equitable relationships in a bioprospecting endeavor (Rosenthal, n.d.). The ICBG Program has explored numerous structures in a broad variety of cultural settings, exploring how benefits related to bioprospecting may be shared across communities and what follow-on impacts these efforts may have on biodiversity conservation (Rosenthal, n.d.).

In 2005 UNESCO issued a Universal Declaration on Bioethics and Human Rights, in which it was stated in Article 17 that, "Due regard is to be given to the interconnection between human beings and other forms of life, to the importance of appropriate access and utilization of biological and genetic resources, to respect for traditional knowledge and to the role of human beings in the protection of the environment, the biosphere, and biodiversity" (UNESCO, 2005). This declaration nicely sums up the needs around the interface between biomedicine and biodiversity, but it unfortunately does not suggest how this daunting goal should be achieved, neither in terms of incentivization, nor in terms of regulation. The fate and impact of the Nagoya Protocol remains to be seen.

Conclusion

Ecological systems tend to become more intricate and complex over time. Biological functions may impact chemical and physical functions, creating an even more delicate balance for life. Often scientists look at these systems as if humans are outside of them, almost as if we and they occupy different planets. And yet, this is not the case. Humans are part of "nearly" every ecosystem on the planet. Much of our impacts are passive, related to pollutants that move through wind and water, or invasive species to which we provide footholds with optimal levels of disturbance. The knowledge base around ecosystems continues to grow. Increasingly we understand and value the complexity of these systems. What remains to be seen is how we will act upon our understanding: will we plunder these systems for the riches they hold, or will we preserve their capacity for adaptation and complexity? Inclusive engagement including youth, religious leaders, ethicists, economists, and other diverse groups of stakeholders may lead to a paradigm shift in our conceptualization of our relationship to the world around us.

The current paradigm used to discuss conservation often couples nature with human benefits in an approach that commodifies nature (and in this case, healthcare). In fact, this book furthers that paradigm. Although emerging solutions will likely have roots in this understanding, it is possible that sustainable management of biodiverse regions of the world will require a rethinking of human–nature relationships. As Grifo made clear decades ago, "Conserving biodiversity requires a fundamental reorganization of the way people think. Every day we make fundamental changes in the long term health of our ecosystem. When we alter or lessen diversity there are ramifications for the entire system, not just the biological factors, but the socioeconomic factors as well. Conserving biodiversity conserves choices" (Grifo, 1995). Indeed, as Hardin (1968) pointed out half a century ago, the error many make when approaching a challenge is thinking that there is a technical solution. When we commodify nature for its benefits we may make the error of relying on the market to provide a working solution. We authors argue that relying on "things to work out" is an abrogation of

our moral responsibility to the current and future generations of Earth's inhabitants. As is the case in so many other complex arenas, sustaining the planet's species diversity may require moral solutions be applied in conjunction with our technical acumen.

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Biodiversity and Biomedicine
Our Future

Examines the diverse connections between conserving Earth's biodiversity and human healthcare

Biodiversity and Biomedicine: Our Future provides a new outlook on Earth's animal, plant, and fungi species as vital sources for human health treatments. While there are over 10 million various species on the planet, only 2 million have been discovered and named. This book identifies modern ways to incorporate Earth's species into biomedical practices and emphasizes the need for biodiversity conservation.

Written by leading biodiversity and biomedical experts, the book begins with new insights on the benefits of biologically active compounds found in fungi and plants, including a chapter on the use of wild fruits as a treatment option. The book goes on to discuss the roles of animals, such as amphibians and reptiles, and how the threatened presence of these species must be reversed to conserve biodiversity. It also discusses marine organisms, including plants, animals, and microbes, as essential in contributing to human health.

Biodiversity and Biomedicine: Our Future is a vital source for researchers and practitioners specializing in biodiversity and conservation studies. Students in natural medicine and biological conservation will also find this useful to learn of the world's most bio-rich communities and the molecular diversity of various species.

- Presents new developments in documenting and identifying species for biodiversity conservation and ethical considerations for biodiversity research
- Examines biodiversity as an irreplaceable resource for biomedical breakthroughs using available species for medical research
- Discusses challenges and opportunities for biodiversity protection and research in biosphere reserves

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