Novel Therapeutic Agents from Plants

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Preface

Since the advent of synthetic drugs, the use of natural products has diminished. However, the diversity of natural molecules still surpasses those from synthetic compounds, and this ensures that natural products will continue to be important for drug discovery. Besides, many of the currently used synthetic drugs are responsible for side-effects and are sometimes extremely costly.

Plants and microorganisms are capable of producing secondary metabolites with a wide range of biological activities which are involved in defensive and protective processes in the hosts. This property of determined organisms should be exploited by man for obtaining new therapeutic preparations or to serve as leading molecules for the synthesis of analogues.

The use of plants in medicine has evolved because, from the biochemical point of view, they produce different chemical compounds with particular modes of action for controlling pathogenic organisms or inhibiting processes involved in many diseases. There are a large number of chemical structures and only a few are well known. The different chemical structures present in a plant -derived extract ensure that target organisms do not develop resistance, and have high effectiveness due to different modes of actions and/or synergism between compounds. Most of these secondary metabolites are harmless, thus ensuring safe clinical use.

A scientific approach to understand the relationship between active chemicals and cure of diseases is recent. More properties and characteristics of the wide range of natural compounds come to light everyday. Such voluminous data has to be compiled and described on the basis of scientific criteria.

While there are several books on natural drugs, the present book covers multiple curative aspects of natural chemicals. This book is a complete

review of medicinally active metabolites produced by nature and looked at from different approaches.

The book describes the effects of natural extracts and/or their isolated compounds and also gives an update of their *in vitro* and *in vivo* effectiveness, active doses, modes of action, production and commercialisation.

María Cecilia Carpinella
Mahendra Rai

1 Anticancer Compounds of Plant Origin

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Introduction

Cancer is a malignant neoplastic disease characterized by uncontrolled growth of cells with the ability of the cells to migrate and spread to distant sites (American Cancer Society, 2006). There are different types of cancers. Carcinomas are cancers that arise in the epithelium (layers of cells covering the body's surface and lining the internal organs and various glands). Ninety percent of human cancers fall into this category. Melanomas are cancers that originate in the skin, usually in the pigment cells (melanocytes) while sarcomas are cancers of the supporting tissues of the body such as bone, muscle and blood vessels. Cancers of the blood and lymph glands are called leukemias and lymphomas respectively, while gliomas are cancers of the nerve tissue.

Cancer is a leading cause of death worldwide. In a total of 58 million

deaths worldwide in 2005, cancer accounted for 7.6 million (or 13 percent) of deaths. The main types of cancer leading to overall cancer mortality include lung (1.3 million deaths/year); stomach (almost 1 million deaths/year); liver (662,000 deaths/year); colon (655,000 deaths/year) and breast (502,000 deaths/year). Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030 (WHO, 2006).

Cancer treatment

The usual treatments for different groups of cancer are surgery, radiation therapy and chemotherapy (treatment with anticancer drugs) or combination of these methods.

Surgery is the main treatment for many types of solid tumors, especially when the cancer has not spread to other parts of the body. This involves surgical removal of all or part of the cancerous tissue. Sometimes it is used in conjunction with chemotherapy and/or radiation therapy.

Radiation therapy involves the use of high-level ionizing radiation to destroy cancer cells. Both tumor cells and healthy cells may be affected by this radiation. Radiation injures the cancer cells and they can no longer continue to divide or multiply. They can be generated either by X-rays or by gamma rays. Other recent radiotherapy research has focused on the use of radio labeled antibodies to deliver doses of radiation directly to the cancer site (radioimmunotherapy).

Chemotherapy is the treatment of cancer with 'anticancer' drugs to destroy cancer cells throughout the body. Chemotherapy may be used to:

- Cure the cancer
- Prevent the cancer from spreading
- Destroy cells that have spread beyond the tumor
- Decrease the size of the tumor
- Relieve symptoms, such as pain

Drugs used in chemotherapy have been classified according to the mechanism of action. It includes alkylating agents like cisplastin and mitomycin; antimetabolites like methorexate, trimetrexate, hydroxyurea and fluorouracil; DNA cutters like bleomycin and DNA binders like dactinomycin. The plant derived or semi-synthetically plant derived drugs that are used in chemotherapy include, taxol, taxotere, vinbalstine, vincristine, vindesine, vinorelbine, teniposide, etaposide, topotecan and irinotecan

Plant Derived Anticancer Drugs

Anticancer drugs derived from plants are believed to have tremendous potential for treatment of various types of cancer. Seven plant based anticancer drugs have received Food and Drug Administration approval for commercial production in the USA (Taylor, 2000). Some of the most studied and marketed anticancer drugs are described below.

Paclitaxel (Taxol®)

Paclitaxel, a diterpene, is derived from *Taxus* spp. belonging to the family Taxaceae, a genus of evergreen trees and shrubs. Seven species of *Taxus* have been recognized and the two major species are *Taxus baccata* Linn. and *Taxus brevifolia* Nutt. *T. baccata*, commonly known as English yew, is native to England, occurring locally in South Scotland, Ireland and Wales. It is also indigenous to central and southern Europe, Algeria and northern Spain and the distribution is primarily based on edaphic conditions. *T. brevifolia* also known as pacific yew, grows in moist soils along the Pacific coast of south eastern Alaska, southward through western British Columbia to central California (Elbert, 1979). It grows as an understory of conifers and has high shade tolerance (Klinka et al., 2000). The species is generally propagated through seeds (Mitiska, 1954) or by vegetative propagation as in *T. baccata* (Mitter and Sharma, 1999) and *T. brevifolia* (Mitchell, 1997). The active components of the tree include taxol and baccatin, isolated from the root bark and needles.

Taxol was first isolated from the bark of *T. brevifolia* by Wall and Wani at the Research Triangle Institute (RTI) in 1967 (Wani et al., 1971) (Figure 1). The broad range antitumor activity of the compound was reported against rodent tumors (Wani et al., 1971). Taxol is the first drug of choice in several tumorous cancers including breast, ovarian, lung cancer, squamosa cell carcinoma, head and neck cancer and AIDS-related Kaposi's sarcoma. In 1992, FDA approved the use of paclitaxel for ovarian cancer that was refractory and in 1994 it was approved for treating breast cancer. In 1997, FDA approved it for treating AIDS-related Kaposi's sarcoma while in 1998 it received approval for use against ovarian and lung cancer. The revenue earned from 1997-1998 by marketing the drug was US$ 45 million while the gross revenue from all the patents in 1997-1998 was around US$ 57.3 million (Eisenstein and Resnick, 2001).

Originally, the only source of Taxol was the Pacific yew but presently the drug is made by a semi-synthetic process. Taxol has been identified in lesser quantities in other *Taxus* sp. like *T. canadensis* Marshall (American yew) (Hezari et al., 1997; Phisalaphong and Linden, 1999), *T. cuspidate* Sieb. (Japanese yew) (Zhang and Su, 2000; Zhang and Su, 2002) and *T. mairei*

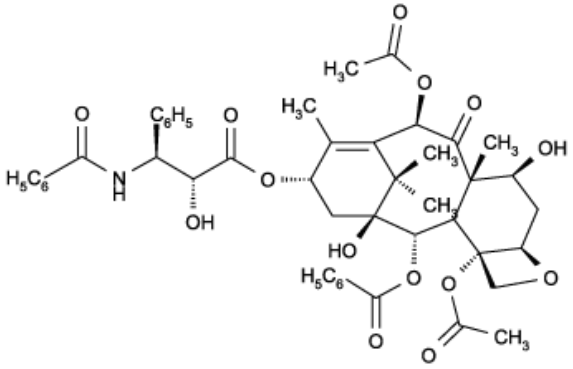

Fig. 1. Structure of Paclitaxel.

Lemée & H. Lév. (Cui and Ge, 2004). Among different *Taxus* species and different tissues of the tree, there is a variable taxol production ranging from zero to 0.069 percent (Castor and Theodore, 1993; Guy et al., 2002). A semi-synthetic compound, Docetaxel (Taxotere®) from *T. baccata* was developed by Rhone-Poulenc Rorer which was similar to paclitaxel. It was more water-soluble than taxol and was produced semi synthetically from 10-deacetylbaccatin III, an inactive precursor, extracted from needles of *T. baccata* (Lavelle et al., 1995). It has been approved as a second-line agent for advanced breast cancer and is found to be more effective than taxol. Other than taxol, a diterpenolignan was also isolated from bark of *T. brevifolia* called brevitaxin, showing cytotoxicity against tumor cell lines (Arslanian et al., 1995).

Studies have shown that endophytic fungi like *Sporormia minima* Auersw. and *Trichothecium* sp. growing in *T. wallichiana* Zucc. produced paclitaxel in culture medium (Shreshtha et al., 2001). Similarly *Tubercularia* sp. growing in *T. mairei* produced taxol in potato dextrose liquid medium.

Three total synthesis of taxol has been reported by Holton Route, Nilcolaou and Danishefsky route and Morihira route (Holton et al., 1994; Nicolaou et al., 1994; Danishefsky et al., 1996; Morihira et al., 1998). However, the complex structure of the compound requires many chemical reaction steps that make commercial-scale production of synthetic taxol unfeasible (Guo et al., 2006).

Mode of action

Taxol binds to β-tubulin subunit of microtubules and promotes assembly of microtubules, stabilizes microtubules by preventing depolymerization and causes formation of stabilized abnormal microtubule bundles (Schiff et al., 1979). It disrupts the equilibrium between free tubulin and

microtubule by shifting direction of assembly and inhibits normal dynamic reorganization of microtubule network that is required for mitotic functions (Ganesh et al., 2004). This novel mode for action made taxol a prototype for a new class of anticancer drugs. The fungal taxol was found to enhance microtuble stability and bundling in culture cells and induced tubulin polymerization *in vitro* similar to the authentic plant taxol (Wang et al., 2000a).

Vinca alkaloids

Vinca alkaloids are derived from a small genus of perennial, evergreen herbs commonly known as periwinkles (divided into 2 distinct groups, treated as separate genera—*Vinca* and *Catharanthus*) which belongs to the family Apocynaceae. The main area within which the species of *Vinca* are native, extends eastwards from Morocco, Algeria, Portugal, Spain and France and over central and southern Europe and southwestern Russia. The genus *Catharanthus* is distributed in Madagascar and India. It is propagated generally through seeds and occasionally by cuttings.

Work on the periwinkle plant, *Catharanthus roseus* Linn. G.Donn. was independently taken up in two different laboratories for its alleged hypoglycaemic activity. The Canadian group of Nobel Beer and Cutts succeeded in isolating vinblastine while Eli Lilly group isolated vinblastine and vincristine along with two other active dimeric alkaloids (Duffin, 2000). These alkaloids were present in extremely low concentrations in a complex mixture of 50 different alkaloids. Vinblastine was introduced (Velban®, Eli Lilly) in 1961 and vincristine (Oncovin®, Eli Lilly) in 1963 as anticancer drugs Figure 2). In India, CIPLA has improved upon the process of isolating vinblastine and vincristine from *C. roseus* as developed by National Chemical Laboratory, Pune (Kaul, 2004).

The vinca alkaloids are considered to be cell phase-specific. It is used frequently in acute lymphatic leukemia, Ewing's sarcoma, Hodgkins's disease, small cell lung cancer, non-Hodgkins's lymphoma, rhabdomyosarcoma, Wilm's tumor, brain tumor, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myelogenous leukemia, colorectal cancer, hepatoblastoma in children, Kaposi's sarcoma, malignant melanoma, multiple myeloma, neuroblastoma, osteogenic sarcoma, soft tissue sarcoma and testicular cancer. A very important feature of vinca alkaloids is their relatively low toxicity.

Vinblastine is an antineoplastic drug and is the first drug of choice in many forms of leukemias and since 1950s it has increased the survival rate of childhood leukemias by 80 percent. It is used for treating cancer of breast, testicles, bladder, kidney, lungs, prostrate and malignant melanoma.

It has a half-life of 24 hours in the bloodstream and is also known to interfere with the glutamic acid metabolism. Vincristine is another antileukemic, antineoplastic drug marketed by Eli Lilly with a half-life of 85 hours in the serum.

Other than vinblastine and vincristine, a new semi-synthetic compound with structural modification from catharanthine unit was synthesized and named vinorelbine (Figure 3). Vinorelbine is effective against non-small cell lung cancer and breast cancer. It is used as a single agent or in combination with cisplatin for the first-line treatment of advanced, unresectable non-small cell lung cancer (NSLC), and is the only single-agent therapeutic to treat NSLC. Another semi synthetic compound derived from vinblastine is Vindesine (Figure 4), which is mainly used to treat melanoma and lung cancer (Huang, 1999).

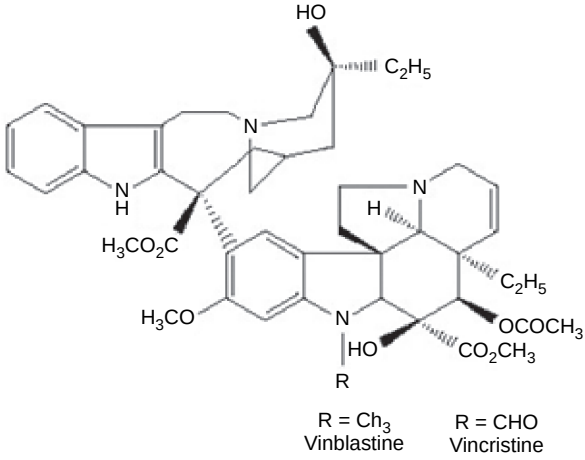

Fig. 2. Structure of Vinblastine and Vincristine.

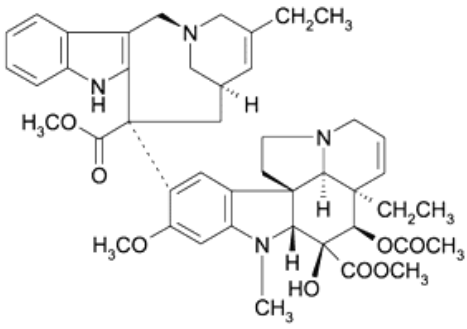

Fig. 3. Structure of Vinorelbine.

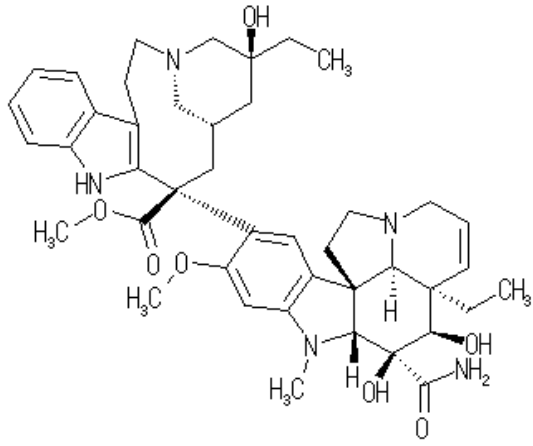

Fig. 4. Structure of Vindesine.

Mode of action

The vinca alkaloids and their derivatives inhibit mitosis in metaphase. They bind to tubulin, thus preventing spindle formation and arresting cell division. They also interfere with the cells' ability to synthesize DNA and RNA.

Podophyllotoxins

Podophyllotoxin and Epipodophyllotoxin are active cytostatic glucosides derived from the rhizomes and roots of May apple plant, *Podophyllum peltatum* Linn. *Podophyllum* belonging to the family of Berberidaceae, is a small genus of herbs distributed in the north temperate regions in Canada and eastern US. Three species have been reported from the Himalayan region of India of which *P. hexandrum* Royle. (syn. *P. emodi* Wallich ex Hook. f. & Thomson) is the source of podophyllotoxin (Figure 5) and a resin, podophylline (Foster 1993). Recent findings concluded that the leaf blades of *P. peltatum* could serve as an alternative and renewable source of podophyllotoxin (Canel et al., 2001; Moraes et al., 2001). Other than *P. hexandrum* and *P. peltatum*, other sources of podophyllotoxin and its analogs were reported from *Linum, Juniperus, Hyptis, Teucrium, Nepeta, Dysosma, Jeffersonia, Thymus and Thuja* (Kupchan et al., 1965; San Feliciano et al., 1989a,b; Broomhead and Dewick, 1990a,b; Yu et al., 1991; Kuhnt et al., 1994; Konuklugil et al., 1996a,b). Other anticancer principles of *Podophyllum* are contained as resins called podophylline. American *Podophyllum* yields about 2-8 percent, while Indian *Podophyllum* yields about 6-12 percent of

the resin. The Indian *Podophyllum* produces higher resin while the American *Podophyllum* produces more peltatins. The podophyllotoxin content in Himalayan mayapple is high (4.3 percent) compared with other species of *Podophyllum*, notably *P. peltatum* (0.25 percent) (Jackson and Dewick, 1984).

Podophyllotoxin is a precursor of semi synthetic compounds like etoposide, teniposide and etophos (Figure 6 & 7). These compounds have been used for the treatment of lung and testicular cancer as well as certain leukemias (Stahelin and Wartburg, 1991; Imbert, 1998). Synthetic studies of podophyllotoxin derivatives are divided into four general approaches—the oxo-ester route, the dihydroxy acid route, the tandem conjugate addition route and the Diels-Alder route (Botta et al., 2001).

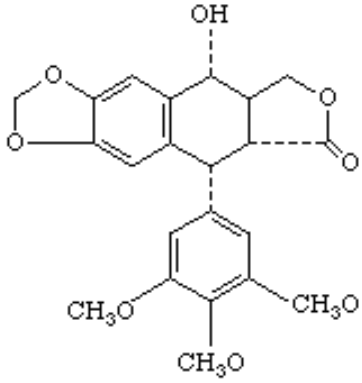

Fig. 5. Structure of Podophyllotoxin.

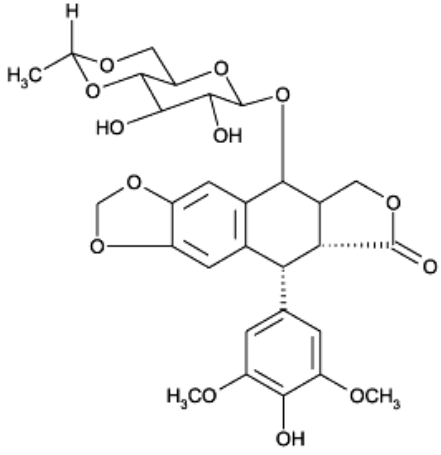

Fig. 6. Strcture of Etoposide.

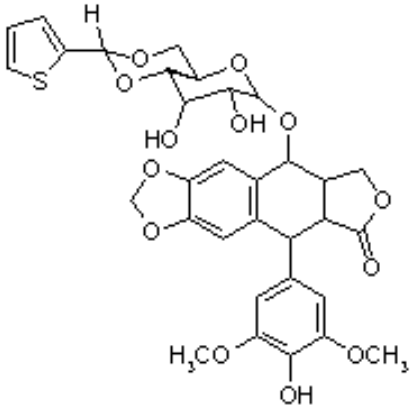

Fig. 7. Structure of Teniposide.

Etoposide is used in the combination therapy for testicular cancer, lung and breast cancer, some lymphomas, acute nonlymphocytic leukemias and Kaposi's sarcoma. It has been tested in 167 clinical trials for the use as a new investigative cancer treatment or as positive control (Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999). Teniposide is antineoplastic and is used to treat acute lymphoblastic leukemia. It is also used for acute lymphocytic leukemia and monocytic leukemia in children, brain tumors in adults, neuroblastoma in children and non-Hodgkin's lymphoma.

Mode of action

Both etoposide and teniposide block the cell cycle in two specific places, between the last division and the start of DNA replication (G1 phase) and also the replication of DNA (S phase), thus preventing cells from entering mitosis. It is known to inhibit topoisomerase II activity and forms stabilized cleavable complex (DNA-topo II drug) leading to DNA double strand breaks which blocks cells at S-G2 interphase.

Camptothecin alkaloids

Camptothecin, a monoterpenoid indole alkaloid (Figure 8), is derived from the bark of *Camptotheca acuminate* Decne. and *Nothapodytes foetida* (Wight) Sleumer. *C. acuminata* belonging to the family Nyssaceae, is a rapidly-growing, deciduous tree native to China and Tibet, where it is known as Xi Shu ('happy tree'). It occurs at elevations from 150m to 2,400m in South-eastern China, and it also grows in Myanmar and northern Thailand. The tree forms part of the Chinese mixed mesophytic forest in warm, moist, temperate regions. *N. foetida* (*Mappia foetida*) renamed as *N. nimmoniana*

Graham. belonging to the Icacinaceae family is a small tree native to warm, broad-leaved forests in India. It has been recorded at an altitude of 1,830 metres in the Himalayan foothills, northern India, at locations including Lopchu and Rungbi, near Darjeeling (Grierson and Long, 1984) and Western Ghats (Padmanabha et al., 2006). It is a far richer source of camptothecin than *C. acuminat*a (where camptothecin content is approximately 0.001 percent) with an average camptothecin content of approximately 0.1 percent. Camptothecin was also isolated from a variety of plant species including *Merriliodendron megacarpum* (Hemsl.) Sleumer (Arisawa et al., 1981), *Ophiorrhiza mungos* Linn. (Tafur et al., 1976), *O. pumila* Champ. (Aimi et al., 1990), *Ervatamia heyneana* (Wall.) T. Cooke and *Mostuea brunonis* Didr. (Gunasekera et al., 1979). Traditionally, camptothecin was extracted from root, root bark and fruits but later its presence was detected in young leaves (Lopez-Meyer et al., 1994; Zhang and Yang, 1997). The camptothecin content in leaves was found to be linearly correlated to the leaf area, climatic factors where high temperature, high evaporation capacity and low precipitation were found to increase its content (Yan et al., 2003).

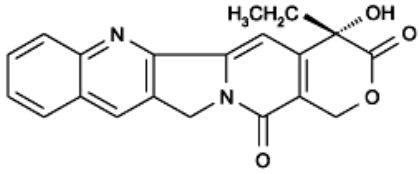

Fig. 8. Structure of Camptothecin.

Its potential as a source of anticancer agent was first noted by Monroe E. Wall and Jonathan Hartwell of National Cancer Institute (Wall et al., 1966). The intact lactone E ring of the camptothecin is known to be essential for its cytotoxic activity. The compound undergoes a reversible pH-dependent hydrolysis of the active lactone form to an inactive ring-opened hydroxy acid anion form. Camptothecins are lauded as one of the most promising anticancer drugs of the 21st century. Topotecan (TPT) (Lilenbaum et al., 1995; Romanelli et al., 1998; Clements et al., 1999) and irinotecan (CPT II) (Masuda et al., 1992; Abigerges et al., 1995; Bleiberg, 1999) are two water soluble derivatives of camptothecins and have gained approval by FDA for treating colorectal and ovarian cancer. Topotecan is an analog of the first generation compound discovered by RTI called 10-hydroxy camptothecin (Figure 9). It was approved by FDA for treatment of ovarian and small cell lung cancers. It is currently in clinical trials, either alone or in combination with other anticancer drugs for several other types of cancer. Irinotecan, another analog of 10-hydroxy camptothecin (Figure

10) was discovered by Daiichi Pharmaceutical Co. Ltd. and was approved by FDA for treatment of metastatic colorectal cancer. It is used for treating refractory ovarian cancer in Japan, non-small-cell lung cancer in Europe and metastatic colorectal cancer in the USA.

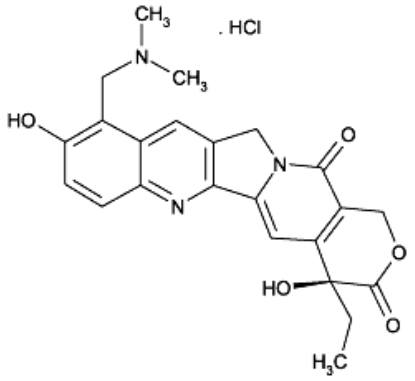

Fig. 9. Structure of Topotecan.

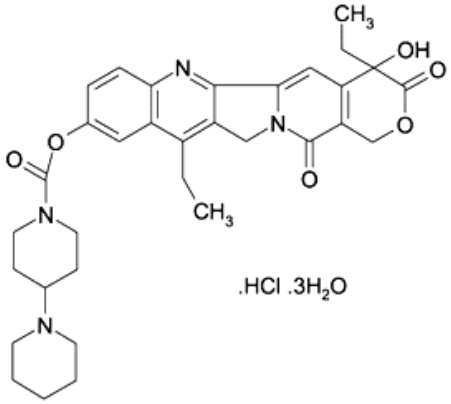

Fig. 10. Structure of Irinotecan.

Other than topotecan and irinotecan, *C. acuminata* is a source of other promising anticancer drugs like 9-aminocamptothecin (9AC), a second-generation analog, presently in clinical trials against ovarian and stomach cancers and T-cell lymphoma, 9-nitro camptothecin (9NC) and 7-(4-methyl piperazino-methylene)-10,11-ethylenedioxy camptothecin (GG211) (Wall and Wani, 1996; Giovanella, 1997; Jeha et al., 1998; Stevenson et al., 1999).

Mode of action

It is a topoisomerase I inhibitor and forms cleavable complex with DNA and topo I causing single-strand breaks (Kohn and Pommier, 2000; Bruschi et al., 2001). Studies have shown that the down regulation of topoisomerase I is via an ubiquitin/26S proteasome pathway (Desai et al., 2001).

A list of potential plant derived anticancer drugs is given in Table 1.

Other than natural or semi synthetic drugs, bioactive compounds in plants are used in Complementary/Alternative medicine (CAM) for prevention and treatment of cancer. CAM can be defined as "diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine" (Ernst et al., 1995). It is immensely popular with about 40 percent prevalence in the USA (Astin, 1998); 50 percent in Australia (MacLennan et al., 1996) and 65 percent in Germany and the overall prevalence of this treatment in 13 countries is about 31 percent (Ernst and Cassileth, 1998). The most popular therapies include dietary treatments, herbalism, homeopathy, hypnotherapy, imagery/visualization, meditation, mega-vitamins, relaxation and spiritual healing (Ernst and Cassileth, 1999). Glycoproteins like sphyrnastatin 1 and 2 from hammerhead shark (Folkman, 1992); mistletoe lectin, viscumin (Kleijnen and Knipschild, 1994; Kaegi, 1998a) thymus extract (Gieldanowski et al., 1987); hydrazine sulphate (Gold, 1968); herbal mix of *Arctium lappa* L, *Rheum palmatum* L., *Rumex acetosella* L. and *Ulmus fulva* Michx. (Kaegi, 1998b); diallyl sulphate found in *Allium* sp. (Schaffer et al., 1996), *Panax ginseng* C.A. Mey (Yun, 1996) and green tea (Wang et al., 1992) have all found their use in CAM.

Even though a high percentage of cancer patients depend on CAM, oncologists have their reservations in the efficacy of these medicines since most of them are not clinically tested. Hence, more reliable treatments include chemotherapy, radiation therapy and surgical oncology.

Other potential sources of anticancer compounds

Several bioactive compounds with cytotoxic and antineoplastic activity have been identified from plants, which could later become a marketable source of anticancer drugs. Some of them have been discussed in the present chapter.

Cephalotaxus harringtonia (Siebold & Zucc.) Koidz belonging to the family Cephalotaxaceae is an endangered species found in Northwest China, Korea and Japan (Tripp, 1995). It is a source of two antitumor esters, harringtonine and homoharringtonine (HHT) and is under clinical trials

Table 1. Plant derived anticancer drugs

Species	*Plant part used*	*Relevant anticancer compound*	*Effectivity against different cancer*	*Trade name*	*Reference*
Taxus brevifolia Nutt.	Bark	Paclitaxel	Ovarian, breast, lung cancer, AIDS-related Kaposi's sarcoma	Taxol®	Cragg et al., 1993; Taylor, 2000
Taxus baccata L.	Needles	Docetaxel	Advanced breast cancer	Taxotere®	Taylor, 2000
Catharanthus roseus (L.) G. Don	Roots/ Leaves	Vinblastine Vincristine Vindesine Vinorelbine	Acute lymphatic leukemia, Edwig's sarcoma, small cell lung cancer, brain tumor, breast, cervical, prostrate bladder, kidney colorectal cancer, hepatobalstoma, malignant melanoma, neuroblastoma, soft tissue sarcoma, osteogenic sarcoma	Velban® Oncovin® Eldisine®, Fildesin® Navelbine®	Huang, 1999; Taylor, 2000
Podophyllum peltatum L.	Rhizomes/ Roots	Podophyllot oxin Teniposide Etapocide	Tesicular cancer, lung and breast cancer, some lymphomas, acute nonlymphatic leukemias, Kaposi's sarcoma	Vumon Vepesid, Etapophos	Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999; Taylor, 2000
Camptotheca acuminate Decne; *N. nimmoniana Graham.*	Root, root bark, fruits and young leaves	Camptothecin Topotecan Irinotecan 9-aminocampt othecin	Ovarian, colon, lung, gastric, non-small cell lung, colorectal, stomach, cancer, leukemias	Hycamtin® Camptosar®	Masuda et al., 1992; Romanelli et al., 1998; Clements et al., 1999; Bleiberg, 1999

by National Cancer Institute, USA against refractory acute non-lymphocytic leukemias, acute promyelocytic leukemia and chronic phase chronic myelogenous leukemia (Kantarjian et al., 2001; O'Brien et al., 2002). The principal mechanism of action of HHT is the inhibition of protein synthesis in a dose-and time-dependent manner by acting on the ribosomes of cancer cells. It blocks the progression of cells from G1 phase into S phase and from G2 phase into M phase (Zhou et al., 1995).

Bruceantin, a triterpene of the quassinoid type was isolated from the bark of the Ethiopian tree *Brucea antidysenterica* Mill belonging to the family Simaroubaceae, was found to be a potential chemotherapeutic agent (Sneden, 1979). Nine additional quassinoids, bruceantarin, bruceantinol, bruceine B, bruceolide, dehydro bruceantin, dehydrobruceantarin, dehydrobruceine B, dehydrobruceantol, and isobruceine B were isolated from the same plant. Bruceantin, bruceantarin, bruceantinol, bruceine B, and dehydrobruceantin were also isolated from the Ghanaian tree *Brucea guineensis* Don. Bruceantin demonstrated significant activity *in vivo* against several tumor systems including lymphocytic leukemia, lymphoid leukemia, adriamycin resistant P388 leukemia, cytoxan resistant P388 leukemia, melanocarcinoma, and the Lewis Lung carcinoma. The mode of action of the compound was irreversible inhibition of protein synthesis and partial inhibition of DNA synthesis (Huang, 1999).

Maytansine was first isolated from the African plant *Maytenus ovatus* (Wall. ex Wight & Arn.) Loes. (*Maytenus serrat*a (Hochst. ex A. Rich.) R. Wilczek) belonging to the family Celastraceae. Later, *M. buchananii* was identified as a better source of maytansine yielding about 1.5 mg/kg. It is also a source of several other metabolites termed as maytansinoides (Larson et al., 1999). The compound showed antileukemic activity and cytotoxicity against the KB cell culture derived from a human epidermoid carcinoma of the mouth. Similar cytotoxic aromatic triterpenes were isolated from *Maytenus ilicifolia* Mart. ex Reissek (Shiroto, 1994). The cytotoxicity of maytansine was attributed to the inhibition in tubulin polymerization resulting in mitotic block and cell death. Its mode of action is similar to the vinca alkaloids but the cytotoxicity of maytansine was 200-1000 fold more than vinblastine and vincristine (Huang, 1999).

Betulinic acid, a pentacyclic triterpene is a novel cytotoxic compound derived from the bark of *Betula alba* L. and *Zizyphus mauritiana* Lam. and is cytotoxic against medulloblastoma and glioblastoma cells. Cancer cells treated with betulinic acid showed enhanced mitochondrial membrane damage leading to apoptosis (Fulda et al., 1999).

Colchicine derived from *Colchicum autumnale* L. exhibit antimitotic properties by interfering with microtubule—dependent cell function and bind to tubulin irreversibly. Demecolcine is less toxic than colchicines and

have been shown as anti leukemia agent (Yamamoto et al., 2001). Colchicine is abundantly present in *Gloriosa superba* L., and *Colchicum autumnale* (Wildman and Pursey, 1968), and is effective against breast, thyroid and esophagus cancer (Huang, 1999).

Combretastin A was isolated from the South African tree *Combretum caffrum* (Eckl. & Zeyh.) Kuntze and is presently in phase I clinical trial. It showed concentration-dependent cytotoxicity against human tumors (El-Zayat et al., 1993). Its prodrug, combretastatin A-4 phosphate was found to be both antitumor and antivascular causing complete shutdown in tumor cells leading to necrosis (Holwell et al., 2002).

The benzophenanthridine alkaloid sanguinarine and the protoberberine alkaloid berberine occur in several genera of the families Papaveraceae (*Agremone, Bocconia, Chelidonium, Dicranostigma, Escholtzia, Glaucium, Hunnemannia, Hylomecon, Macleaya, Meconopsis, Papaver, Platystemon, Romneya, Sanguinaria, Stylomecon and Stylophorum*), Berberidaceae (*Berberis and Mahonia*), Fumariceae (*Corydalis*), Hypococaceae (Hypecoum) and less abundantly in Menispermaceae (*Jateorhiza*), Ranunculaceae (*Thalictrum*), Rutaceae (*Zanthoxylum*) and Sapindaceae (*Pteridophyllum*). These alkaloids bind to microtubules, inhibit several enzymes, including Na+, K+-ATPase, uncouple oxidative phosphorylation and intercalate in GC-rich regions of DNA (Krey and Hahn, 1969; Smekal and Kubova, 1984). Sanguinarine is a potent inhibitor of the nuclear factor NF-kappa B activation (Chaturvedi et al., 1997). Berberine has been reported to reduce the *in vitro* growth of brain tumor cells, teratocarcinoma cells and HepG2 cells.

Hadi et al. (2000) have shown that several plant derived polyphenolic compounds can possess anticancer and apoptosis-inducing activity in cancer cells. Evidence shows that polyphenols such as gallotannins, curcumins and resveratrol can have cytotoxic activity against cancer cells that are involved in mobilization of endogenous copper and the consequent prooxidation. Curcumin (deferlolylmethane) is a polyphenol derived from *Curcuma zedoaria* (Christm.) Roscoe, *C. aromatica* salisb., *C. kwangsiensis* S.G. Lee et C.F. Liang and *C. longa* L. (turmeric). It suppresses tumor inhibition, promotion and metastasis. Pharmacologically it was found safe even at higher concentrations of 10g/day (Aggarwal et al., 2003). The antiproliferative effect of this compound was tested against several breast tumor cell lines caused due to arrest of G2/S phase of cell cycle (Mehta et al., 1997). It is also effective against ovarian carcinoma, skin cancer, genital carcinoma, malignant lymphoma, primary hepatoma thyroid cancer, gastric carcinoma, and lung carcinoma. Its chemopreventive activity against induction of tumors in various target organs was also documented (Iqbal et al., 2003). Resveratrol is a cancer preventive agent found in grapes and

red wine (Jang et al., 1997). The antileukemic activity of the compound was attributed to its conversion into piceatannol, a phytoestrogen that triggered cell death in cancer cells (Potter et al., 2002).

The dried aerial parts of *Rabdosia rubescens* (Hemsl.) Hara (Labiatae) are widely used in the province of Hunan, China, to treat esophageal cancer. Several terpines, including rubescensine B, oridonin and ponicidine, are probably responsible for the antitumor activity of the herb (Huang, 1999).

The stigmas of the flowers from *Crocus sativa* L. (Iridaceae) contain several carotene derivatives and glycosides including crocin and picrocrocin. Crocin and dimethyl-crocetin show a potent cytotoxic activity against human cancer cells by disrupting DNA-protein interaction. (Huang, 1999).

Ipomeanol, a pneumotoxic furan derivative produced by sweet potatoes *Ipomoea batatas* (L.) Lam., infected with the fungus, *Fusarium solani*, has been in clinical trials for treatment of lung cancer (Kinghorn and Balandrin, 1993).

Limonene (1-cyclohexene-1-methyl-4-isopropenyl) is one of the most abundant naturally occurring monocyclic monoterpenes found in oils of citrus fruit peel. A number of mechanisms of limonene action have been suggested including induction of carcinogen metabolizing enzymes (Maltzman et al., 1991), growth factor/growth factor receptor expression (Jiritle et al., 1993), inhibition of 3-hydroxy-3-methylglutaryl CoA reductase and inhibition of Ras protein farnesylation (Crowell et al., 1991) suggesting its cytotoxic nature.

Falcarinol, also named panaxynol, is a common constituent of many plants especially *Panax ginseng* belonging to the family Araliaceae. Bioassays have shown that falcarinol has selective *in vitro* cytotoxicity against L1210, MK-1, B-16 and other cancer cell lines (Zheng et al., 1999).

Ukrain is a semisynthetic compound consisting of alkaloids from *Chelidonium majus* L. (Papaveraceae) conjugated to thiophosphoric acid showed antineoplastic and immunomodulatory properties (Nowicky et al., 1987; Liepins and Nowicky, 1996; Jagieto-Wojtowicz et al., 1999). The drug interferes directly with the metabolism of cancer cells.

Criptolepine hydrochloride is an indoloquinoline alkaloid isolated from the roots of *Cryptolepis sanguinolenta* (Lindl.) Schltr. (Periplocaceae). The alkaloid binds tightly to DNA and behaves as a typical intercalating agent. The drug interacts preferentially with GC-rich sequences. Investigations also reveal that cryptolepine is a potent inhibitor of topoisomerase II and a promising antitumor agent (Nowicky et al., 1996; Jagieto-Wojtowicz et al., 1999).

Recently, it has been demonstrated that some indole alkaloids isolated from the root bark of *Alstonia macrophylla* Wallich ex G. Don possessed a pronounced cytotoxic activity against different human tumor cell lines—MOR-P (lung adenocarcinoma), COR-L23 (large cell carcinoma of the lung), StMII Ia (melanoma), Caki-2 (renal cell carcinoma), MCF7 (breast adenocarcinoma), and LS174T (colon adenocarcinoma) (Keawpradub et al., 1999).

Cui et al. (1999) identified 10 cytotoxic compounds composed of 5 novel xanthanolide derivatives, a novel nerolidol derivative, 3 sesquiterpene lactones and hispidulin, a flavonoid from *Ratibida columnifera* (Nutt.) Woot. and Standl. The cytotoxicity of one of the sesquiterpene lactone was studied in detail and was found to induce G1 arrest during cell division.

A diphyllin glycoside called cleistanthin was isolated from the tropical plant *Cleistanthus collinus* (Roxb.) Hook. f. The compound showed preferential cytotoxicity against several tumor cell lines and was found most effective for oral carcinoma cell lines and cervical carcinoma cell lines. It is less toxic compared to other drugs. This compound inhibited DNA synthesis and cell division and caused vigorous membrane blebbing resulting in apoptosis (Kumar and Shanmugam, 1999).

An antitumor/antimetastatic compound called Cassiagrol A was purified from the heartwood of *Cassia garrettiana* Craib. It inhibited the plasmin activity and caused formation of tubes (angiogenesis) in tumor cell (Kimura et al., 2000).

A powerful antineoplastic agent was isolated from bulbs and roots of *Hymenocallis littoralis* (Jacq.) Salisb. (*Pancratium littorale* Jacq.) termed pancratistatin (Pettit et al., 2000), which was found to be toxic to leukemia, ovary sarcoma, melanoma, brain, colon, lung and renal cancers.

Gossypol derived from cotton seeds has antiproliferative and antineoplastic effects on tumor cell lines derived from testis, lung, breast, cervix, melanoma, colorectal carcinoma, and others (Blackstaffe et al., 1997; Wang et al., 2000b). Its cytotoxicity to the tumor cells has been attributed to its telomarase inhibitor activity (Mego, 2002).

Ginkgetin and isoginkgetin are biflavonoids identified from *Ginkgo biloba* L. with anti-proliferative activity (Lee et al., 1995) against human ovarian cancer, breast cancer and hepatocellular cancer (Chao and Chu, 2004).

Lapachol derived from *Tabebuia barbata* (E. Mey) Sandw. belonging to Bignoniaceae, is known to have antitumor activity against laryngeal epidermoid carcinoma. It activates DNA unwinding activity of topoisomerase I b and also enhances the cytotoxic effects of DNA damaging agents and induces DNA strand incisions (Colman de Saizarbitoria et al., 1997; Pinto et al., 2000).

Glycine max (L.) Merr. is a source of an isoflavone called Genistein derived from phenylalanine through the Phenyl propanoid pathway. It shows cytotoxicity against breast and prostrate cancer. It inhibits angiogenesis and interacts with topoisomerase regulating cellular proliferation (Barnes, 1995).

Several other plant compounds have also been found to have anticarcinogenic/antiproliferative effect like alkaloids from *Sedum sarmentosum* Bunge (Kang et al., 2000) and *Peganum harmala* L. seeds (Lamchouri et al., 1999); *Brassica olearaceae* L. var *capitata* extract (Isbir et al., 2000; Van Poppel et al., 2000); prenylflavanones from *Sophora tomentosa* L. and *S. moorcroftiana* Benth. (Shirataki et al., 2001); sesquiterpene helenalin from *Helenium autumnale* L. (Dirsch et al., 2001); Ursolic acid from *Hyptis capitata* Jacq. (Andersson et al., 2003); diterpene acids pseudolaric acid A and B from *Pseudolarix kaempferi* (Lamb.) Gordon. (Pan et al., 1990); bufadienolide called Bryophillin B from *Bryophyllum pinnatum* (Lam.) Oken (Yamagishi et al., 1989); Rottlerin-like phloroglucinol derivatives of *Mallotus japonicus* Thunb. ex L.f. Müll (Arisawa, 2003); cantharidin from *Mylabris* sp. (Wang, 1989); dicine N-oxide (a pyrrolizidine alkaloid) from *Heliotropium indicum* L., ellipticine (a monomeric indole alkaloid) from several *Ochrosia* species (Appendino, 1993), baccharin from *Baccharis megapotamica* Spreng. (Kupchan et al., 1976) and herbal formulation like 'muthu marunthu' made from eight plants (Palani et al., 1999). Other promising agents include *Ostodes paniculata* BI, *Peddiea fischeri* Engl, *Soulamea soulameoides* (A. Gray) Noot. (Handa et al., 1983a,b,c), *Dirca occidentalis* A. Gray. (Badawi et al., 1983); *Passerina vulgaris* Thoday (Guo et al., 1984), indirubin from *Baphiacanthus cusia* (Nees) Brem., *Indigofera tinctoria* Linn., *Polygonum tinctorium* Lour., *Isatis tinctoria* Linn. (Dharmananda, 1991) and rhein from *Rheum* sp. (Losi et al., 1993).

A traditional remedy consisting of an aqueous extract of mixed parts of the tree *Abies alba* Mill. and its mistletoe *Viscum album* L. was tested on benzo ∝ pyrene (BaP) induced tumors in Wistar rats and L-1210 malignant cell lines. The results proved that the extract showed anticarcinogenic/antiproliferative effects due to lectins and thionins contained in *Viscum album* and the monoterpenes contained in *Abies alba* (Karkabournas et al., 2000).

A list of anticancer compounds with potential anticancer properties are listed in Table 2.

Table 2. Anticancer compounds of plant origin

Species	*Plant part used*	*Relevant anticancer compound*	*Effectivity against cancer*
Tabebuia barbata (E. Mey) Sandw., *T. impetiginosa* (Mart. ex DC.) Standl., *T. avellanedae* Lor. ex Griseb.	Heartwood	Beta lapachone, lapachol	Cancer of lung, breast, colon, prostrate, malignant melanomas laryngeal epidermoid carcinoma
Betula alba L.	Bark	Betulinic acid	Melanomas, malignant brain tumor
Colchicum autumnale L.	Rhizome	Colchicine Demecolcine	Antileukemic
Cephalotaxus harringtonia (Knight ex Forbes) K. Koch	Root and rhizome	Homoharringtonine, harringtonine, isoharringtonine	Colon tumors, melanoma, leukemia, adenocarcinoma
Cassia garrettiana Craib	Heartwood	Cassigarol A	Lung cancer
Gossypium sp.	Seeds	Gossypol 1a	Testicular cancer
Cleistanthus collinus (Roxb.) Hook. f.		Cleistanthin	Oral, cervical carcinoma
Brucea antidysenterica Mill. *Brucea guineensis* G. Don	Bark	Bruceantin	Leukemia, melanocarcinoma, Lewis lung carcinoma
Maytenus ovatus (Wall. Ex Wt & Arn.) Loes, *Maytenus buchanani*i (Loes) R. Wilezck	Root bark	Maytansine	Epidermoid carcinoma
Hymenocallis littoralis (Jacq.) Salisb.	Bulb and roots	Pancratistatin	Leukemia, ovary sarcoma, melanoma, brain, colon, lung and renal cancers
Citrus sinensis L.	Fruit peel	Limonene	Mammary carcinoma breast, colon, liver, skin and pancreatic tumors

Contd.

Table 2 continued

Species	*Plant part used*	*Relevant anticancer compound*	*Effectivity against cancer*
Ipomoea batatas (L.) Lam.	Roots infected with *Fusarium solani*	Ipomeanol	Lung cancer, hepatocellular carcinoma
Crocus sativus L.	Stigma, corm	Crocin and dimethyl-crocetin	Ovarian and breast cancer, fibrosarcoma, osteosarcoma
Rabdosia rubescens (Hemsl.) Hara	Dried aerial parts	Rubescensine B, oridonin, ponicidine	Esophageal cancer
Chelidonium majus L.	Roots and rhizomes	Ukrain	Leukemia, bladder cancer, melanoma, prostrate and breast cancer
Glycine max (L.) Merr., *Trifolium pretense* L.	Beans	Genistein, daidzein	Breast and prostrate cancer
Ginkgo biloba L.	Leaves	Ginkgetin and isoginkgetin	Ovarian cancer, breast cancer and hepatocellular cancer

FUTURE PERSPECTIVES

Presently the rate of propagation of pharmacologically relevant plant species in nature is far less than the rate of its exploitation. Research towards either genetically improving the species for higher production of the active component or increasing the production of the compounds in *in vitro* culture conditions is essential. The genetic improvement program is directed on identifying and propagating high yielding genotypes for the active compound. Such studies have been reported in *Podophyllum peltatum* where 28 wild populations were sampled for the podophylotoxin content, which ranged from 0 to 23.6 mg/g (Moraes et al., 2001). Genetic diversity studies aimed at *ex situ* conservation of the species also has been attempted (Bhadula et al., 1996; Singh et al., 2001). However, *ex situ* conservation was found to cause decline in podophyllotoxin content in rhizomes and roots of *P. hexandrum* (Sharma et al., 2000).

Further, studies in genotype-environment interactions on increasing the content of the active principle in vegetatively propagated species are essential. A classical example of this was demonstrated by Idso et al. (2000) in *Hymenocallis littorale*, where increase in atmospheric CO_2 by 75 percent

increased the bioactive component pancratistatin in the bulbs.

Another alternative is to enhance the production of the active principles under *in vitro* culture conditions followed by their effective extraction. The concentrations of the active components in plants are generally low (Table 3) because of slow growth rate and high susceptibility to geographical and environmental conditions. In addition, the *in vitro* culture method affords versatile capabilities for tailoring the chemical structures to maximize their beneficial effects. Most of the research in this field has been directed towards tissue culture of *Taxus* spp., *Catharanthus roseus, Podophyllum peltatum, Cephalotaxus harringtonia* and *Camptotheca acuminata*.

Table 3. Concentration of antitumor compounds in plants

Antitumor compound	*Dry weight (%) in plants*
Baccharin	2.0×10^{-2}
Bruceantin	1.0×10^{-2}
Camptothecin	5.0×10^{-3}
Ellipticine	3.2×10^{-5}
Homoharringtonine	1.8×10^{-5}
Maytansine	2.0×10^{-5}
Podophyllotoxin	6.4×10^{-1}
Taxol	5.0×10^{-1}
Tripdiolide	1.0×10^{-3}
Vinblastine, vincristine	5.0×10^{-3}

Source: Plant tissue culture: an alternative for production of useful metabolite, Misawa, 1994.

Alternative ways of production of taxol, other than extraction from bark, include plant tissue and cell cultures as well as chemical transformation processes from baccatin extracted from needles of the *T. baccata* (Gueritte-Voegelein et al., 1986). Shuler et al. (1994) reported that a cell line of *T. brevifolia* in suspension produced 3.9 mg/L taxol in medium after 26 days of culture and the total taxol produced was secreted into the medium. Similar studies in other *Taxus* species for overproduction of taxol is widely documented (Flores et al., 1993; Wickremesinhe and Arteca, 1993, 1994; Frett-Neto et al., 1994, 1995; Srinivasan et al., 1996; Cheng et al., 1996; Hezari et al., 1997; Goleniowski, 2000; Guy et al., 2002; Hu et al., 2003; Ho et al., 2005). Several factors are known to enhance the taxol production under culture conditions like selection of suitable cell lines (Chang et al., 1996), higher concentration of sugar and fructose (Kim et al., 1995) and induction by using methyl jasmonate (Yukimune et al., 1996) and fungal

elicitors (Ciddi et al., 1995), arachidonic acid (Srinivasan et al., 1996), silver ion (Zhang et al., 2000), chitosan (Zhang et al., 2000; Liden and Phisalaphong, 2000), La^{3+} ion (Wu et al., 2001) and ethylene inhibitors (Zhang and Wu, 2003). It was also reported that haploid and haploid-derived cultures of cells from any species of *Taxus* produced taxanes. Most significantly, female gametophyte tissues taken from immature seeds produced significant quantity of taxanes under culture conditions (Durzan and Ventimiglia, 1994).

Vinbalstin and vincristin, produced commercially by extraction from *Catharanthus roseus* is reported to be present in very low concentration (0.0005 percent as dry weight basis) in plants. Misawa (1994) studied the production of vinblastine by alternative way and established the production of catharanthine by plant cell fermentation and simple chemical or enzymatic coupling. Similar studies on catharanthine production from the root cultures of *C. roseus* was also reported (Jung et al., 1992; Vazquez-Flota et al., 1994; Moreno et al., 1995; Rijhwani and Shanks, 1998).

Reports are also available on tissue culture of *Podophyllum peltatum* and *Juniperus chinensis* for the production of podophyllotoxin (Moraes-Cerdeira et al., 1998; Muranaka et al., 1998; Petersen and Alferman, 2001), *Cephalotaxus harringtonia* for the production of homoharringtonine, harringtonine and isoharringtonine (Westgate et al., 1991; Wickremesinhe and Arteca, 1993; Janick et al., 1994), *Camptotheca acuminata* and *Nothapodytes foetida* for camptothecin (Jain and Nessler, 1996; Ciddi and Shuler, 2000; Fulzele et al., 2002) and pancratistatin from *Hymenocallis littoralis* (Backhaus et al., 1992). Although tissue culture of these plants is a lucrative source of active component for the pharmaceutical industry, very limited success has been achieved in increasing the content of the active principle in culture conditions (Misawa, 1994).

Genetic improvement of species like *Taxus* spp. through conventional breeding strategy is mainly limited by the slow growth rate, long generation time of the species and high degree of heterozygosity in the genome. Genetic engineering promises to augment the *in vitro* production of the active principle by direct manipulation of the genes encoding the key enzymes in the biosynthetic pathway. This will lead to a much higher yield of active compounds either in *in vitro* cultivation of tissues or in regenerated plants expressing the genes in question at higher levels. Genetic transformation in *T. brevifolia, T. baccata* and *T. mairei* was reported by Han and coworkers (Han et al., 1994, 1999; Ho et al., 2005). Research is also in progress to identify key enzymes and their corresponding genes involved in the metabolic pathway as achieved by identification and cloning of the gene encoding taxadiene synthase, the cyclization enzyme in the taxol pathway (Hezari et al., 1995; Wildung and Croteau, 1996; Wu

et al., 1999). Identification of such target genes will lead to a generation of transgenics with increased yield of the secondary metabolite.

Thus, genetic improvement of pharmacologically important plants, their propagation through seeds and vegetative means, *in vitro* culture protocols to enhance the production of secondary metabolites, biochemical pathway engineering for increasing the production of active principles, need to be researched and developed for formulation of drugs for combating various kinds of carcinomas

Conclusion

Tropical forests have been the source of 60 percent of the anticancer drugs discovered in the last 10 years and offers a US$ 200 billion market for plant derived drugs and a potentially huge economic reason for preserving the forests. Analysis of the FDA approved drugs in United States in a span of 12 years (1983-1994) showed that 157 drugs out of 520 drugs approved (30 percent), were natural products and their derivatives (Cragg et al., 1997a) and about 61 percent of the anticancer agents approved were natural products and their derivatives. Nature has provided many of the effective anticancer agents in current use, such as the microbially derived drugs, dactinomycin, bleomycin, and doxorubicin, and plant-derived drugs like vinblastine, irinotecan, topotecan, etoposide, and paclitaxel (Cragg et al., 1997b). While the chemo synthesized drugs are often more potent than neutraceuticals, they are expensive, toxic and less eco friendly. Hence enhanced research on promising plant products towards sustainable development of anticancer drugs can help in continuous availability of these products to the society.

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2 Antimicrobial and Antiviral Metabolites from Polypore Fungi

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Introduction

Polypores and corticioid fungi are members of the Aphyllophorales, a group of morphologically complex, terrestrial basidiomycetes. A phylogenetic classification for these fungi is under development, but the groups are probably not monophyletic (Hibbet and Thorn, 2001). As a result of updates in nomenclature and systematics information over the last 30 years, escalating even more from DNA sequence analyses, there are numerous changes in the names of these fungi to reflect the phylogenetic situation. This causes a problem in evaluating the older literature and in comparing studies of the same fungus that has been known by one or more different names. The fungal names used in this chapter generally follow those of Gilbertson and Ryvarden or other recent monographic materials (Gilbertson and Ryvarden, 1986, 1987; Kirk et al., 2001; Index Fungorum database, 2004).

Many polypores are saprobic wood decayers, and as such these fungi are most often found on logs, stumps, or other dead wood. They are typically tough and woody, and produce basidiospores on walls of tubes of the undersurface hymenophore (the tissue that bears the fertile layer). Common names for the fruiting bodies or basidiocarps of polypores include conks, shelf, and bracket fungi. Some basidiocarps are perennial and others often do not rot readily; they may remain undecayed to the

point that algae or mosses begin to grow on their surfaces. As mentioned above, the majorities of polypores absorb nutrients from the dead woody plant parts and as such are saprobic. These may include polypores that grow on living trees and cause decay of the nonfunctional heart wood. A few of these fungi invade conducting plant tissues and as such are parasites; a few others are mycorrhizal and exchange nutrients and carbon with the roots of plants.

Several excellent review articles have been published on the subject of biologically active metabolites from basidiomycetes (Ayer and Browne, 1981; Anke, 1989; Vidari and Vita-Finzi, 1995; Lorenzen and Anke, 1998; Wasser and Weis, 1999a,b,c; Brandt and Piraino, 2000; Abraham, 2001; Reshetnikov et al., 2001; Stamets, 2001; Smith et al., 2002a,b; Stamets, 2002; Wasser, 2002a,b; Chang and Buswell, 2003; Smith et al., 2003; Lindequist et al., 2005; Lull et al., 2005; Petrova et al., 2005; Zaidman et al., 2005; Fan et al., 2006; Quang et al., 2006; Piraino, 2006; Sullivan et al., 2006; Moradali et al., 2007; Xiao et al., 2007; Zjawiony, 2007). This chapter focuses exclusively on polypores, which are considered by many authors as a major source of pharmacolo-gically active natural products. The primary and secondary metabolites of polypores exhibit a wide range of biological activities such as antimicrobial, antiviral, anticancer, cardiovascular, anti-inflammatory, antioxidant, immunostimulating, nematocidal, and other activities (Stamets, 2002; Zjawiony, 2004). The antimicrobial and antiviral activities of metabolites from polypores are the most commonly observed in this group of fungi.

Antibacterial and antifungal secondary metabolites

According to biological evaluation of over 200 mushroom species, more than 75% of screened polypores showed strong antimicrobial activity (Suay et al., 2000) In another qualitative screening an even higher ratio of antimicrobial activity for tested polypores has been shown (Keller et al., 2002). These activities are associated not only with small molecule secondary metabolites but also with high molecular weight cell-wall polysaccharides.

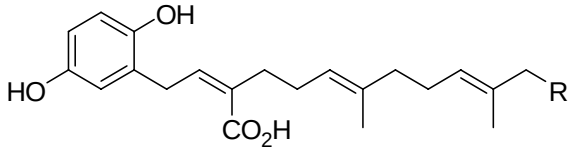
The major philosophy of the search for antimicrobial compounds from basidiomycetes is that humans and animals share common microbial pathogens with fungi, such as *Escherichia coli*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa*. Thus, humans may benefit from defensive strategies used by fungi against microorganisms, also explaining the fungal origins of many antibiotics.

Basidiomycetes, especially polypores, have a long history of medicinal use. For instance, the tinder polypore, *Fomes fomentarius*, was used in the 18th and 19th centuries as haemostatic dressings and bandages (Roussel

et al., 2002). The same polypore together with the birch polypore (*Piptoporus betulinus*), that has had a variety of medicinal and other uses, was found with the body of the famous 5,300 years old 'Ice Man' in a glacier of the Italian Alps in 1991. It is not known, however, how he used either of these fungi (Peintner et al., 1998; Stamets, 2002).

There are numerous publications describing antimicrobial properties of secondary metabolites isolated from various polypores (Robbins et al., 1947; Locquin et al., 1948; Birkinshaw et al., 1952; Marcus, 1952; Takeuchi et al., 1969; Takeuchi et al., 1971; Mellows et al., 1973; Mantle and Mellows, 1973; Nair and Anchel, 1975; Nakajima et al., 1976; Nair and Anchel, 1977; Deol et al., 1978; Giannetti et al., 1978; Quack et al., 1978; Kupka et al., 1981; Anke et al., 1982; Lauer et al., 1989; Clough, 1993; Abate and Abraham, 1994; Dagne et al., 1994; DeJong et al., 1994; Anke et al., 1995; Keller et al., 1996; Smania et al., 1997; Smania et al., 1998; Smania Jr. et al., 1999; Gerber et al., 2000; Mothana et al., 2000; Aqueveque et al., 2002; Kawagishi et al., 2002; Morrison et al., 2002; Angawi et al., 2003; Kamo et al., 2003; Levy et al., 2003; Wang et al., 2004; Chu et al., 2005; Gao et al., 2005; Hashimoto et al., 2005; Luo et al., 2005; Jin and Zjawiony, 2006; Wang and Ng, 2006).

Screening of crude extracts of Ganoderma basidiocarps, such as *Ganoderma lucidum* (reishi mushrooms), *G. pfeifferi*, and *G. resinaceum*, revealed selective activity against *Bacillus subtilis*.

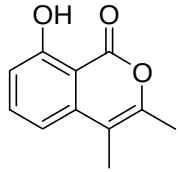


ganomycin A (**1**) R=OH
ganomycin B (**2**) R=H

For example, two secondary metabolites, ganomycins A (**1**) and B (**2**), isolated from *G. pfeifferi* showed moderate growth inhibition of several bacterial strains, particularly Gram-positive strains such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus flavus* (Mothana et al., 2000).

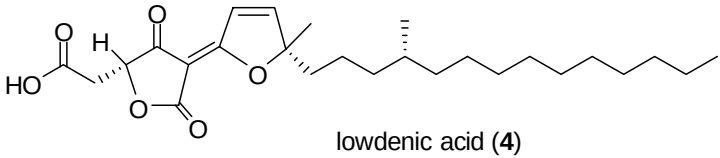
The antifungal isocoumarin, oospolactone (**3**), was identified as a secondary metabolite of *Gleophyllum sepiarium* (Nakajima et al., 1976). This compound was most active against strains of the asexual ascomycete *Alternaria*, showing MIC values of 12.5-25 μg/mL (66-132 μM).[1]

[1]MIC or IC_{50} values wherever possible were recalculated by the author of this article to μM concentrations to facilitate comparison of biological activity of metabolites.



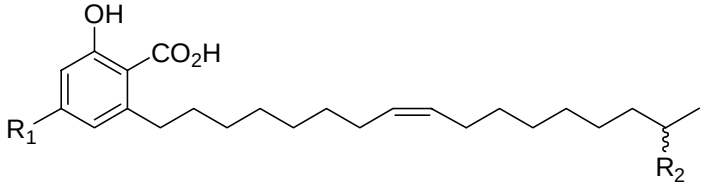
oospolactone (**3**)

An antifungal polyketide-derived secondary metabolite, lowdenic acid (**4**), was obtained from non-sporulating cultures of *Verticillium* species isolated from basidiomata of a polypore *Poria* sp. (Coriolaceae) (Angawi et al., 2003). Lowdenic acid (**4**) is active against *Aspergillus flavus* with an MIC value of 6 µg/mL (13 µM), and also inhibits the growth of *Candida albicans, Staphylococcus aureus*, and *Bacillus subtilis* in the standard disk zone inhibition assay (Angawi et al., 2003).



lowdenic acid (**4**)

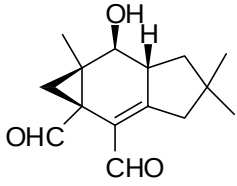
Another group of antimicrobial metabolites of polyketide origin are merulinic acids A, B, and C (**5-7**) isolated from the fruiting bodies of polypores *Merulius tremellosus* and *Phlebia radiate* (Giannetti et al., 1978).



merulinic acid A (**5**) R_1=OH, R_2=H
merulinic acid B (**6**) R_1=H, R_2=OH
merulinic acid C (**7**) R_1=H, R_2=H

The merulinic acids showed antimicrobial activity with MIC values of 0.4-10 µg/mL (1-24 µM), particularly against *Arthrobacter citreus, Bacillus subtilis, Corynobacterium insidiosum, Micrococcus roseus*, and *Sarcina lutea*. *Mycobacterium phlei* was selectively inhibited by **6** and **7**, while **5** was inactive. Similarly, *Staphylococcus aureus* and *Proteus vulgaris* were inhibited only by merulinic acid B (**6**). It is interesting that mycelial cultures of *Merulius tremellosus* do not produce merulinic acids, but instead a highly

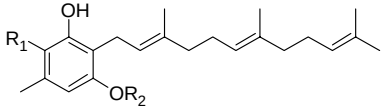
antifungal sesquiterpenoid, merulidial (**8**) (Giannetti et al., 1978). This occurrence serves as an example of the influence of different life cycles stages on the production of fungal secondary metabolites.



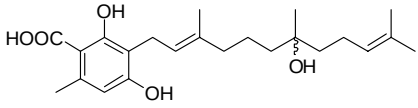
merulidial (**8**)

Biological activities of merulidial (**8**) are associated with the presence of two aldehyde functions. The triol obtained by the reduction of merulidial is inactive (Quack et al., 1978).

Structurally similar to merulinic acids, the antifungal grifolin (**9**), grifolic acid (**10**), grifolic acid methyl ether (**11**), and grifolinol (**12**), were isolated from *Albatrellus dispansus* (Hashimoto et al., 2005).

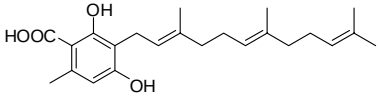


grifolin (**9**) R_1=H, R_2=H
grifolic acid (**10**) R_1=COOH, R_2=H
grifolic acid methyl ether (**11**) R_1=COOH, R_2=Me



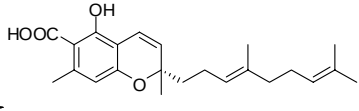
grifolinol (**12**)

One-step reaction of grifolic acid (**10**) with DDQ produced a racemic mixture of daurichromenic acids (**13** and **14**). This was resolved separated into the individual enantiomers by chiral HPLC.

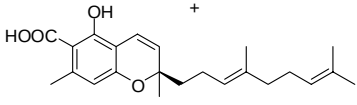


grifolic acid (**10**)

1. DDQ
2. chiral HPLC



(+)-daurichromenic acid (**13**)



(-)-daurichromenic acid (**14**)

Compounds **9**, **10**, **11** and two semi-synthetic enantiomeric daurichromenic acids (**13** and **14**) have shown significant growth inhibition against

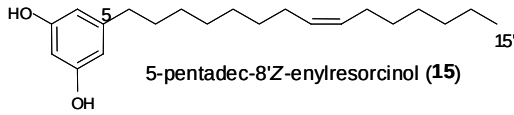
five bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enteriditis* and two fungal species (*Aspergillus niger* and *Candida albicans*). In the standard disk zone inhibition assay all of these compounds showed up to three-fold larger zones of growth inhibition than corresponding controls (gentamycin for bacteria and nystatin for fungi). Grifolin (**9**) and grifolic acid (**10**) were also active against the Gram-positive bacteria *Bacillus subtilis* and *B. brevis* (Zechlin et al., 1981).

A crude, ethanolic extract of the fruiting bodies of *Albatrellus dispansus* from Yunnan province in China and its major active metabolite grifolin (**9**) have shown antifungal activity against plant pathogenic fungi (Luo et al., 2005). Antifungal activities of grifolin were evaluated *in vitro* against nine plant pathogenic fungi and *in vivo* against the wheat powdery mildew *Erysiphe graminis* (Luo et al., 2005). *In vitro*, *Sclerotina sclerotium* and *Fusarium graminearum* were the most sensitive fungi to grifolin and their mycelial growth inhibition were 86.4 and 80.9% respectively, at a concentration of 304.9 μM of grifolin. Spore germination of *F. graminearum*, *Gloeosporum fructigenum* and *Pyricularia oryzae* was almost completely inhibited by a 38.1 μM solution of grifolin. *In vivo*, the curative effect of grifolin against *Erysiphe graminis* was 65.5% at 304.9 μM concentration after an eight day treatment.

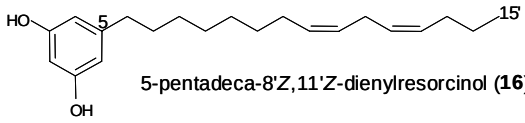
A series of methacilin-resistant *Staphylococcus aureus* (MRSA) inhibiting 5-alkylresorcinols (**15-20**) with C-15 (**15-16**) and C-17 (**17-20**) unsaturated side chain was isolated from *Merulius incarnatus* (Corticiaceae) (Jin and Zjawiony, 2006). Compound **19** was the most active, with an IC_{50} value of 2.5 μg/mL. Compounds **15-18** and **20** were moderately active, with IC_{50} values of 9.5, 5.0, 8.0, 6.5, and 15 μg/mL (30, 16, 23, 19, and 44 μM), respectively, as compared to ciproflaxin (IC_{50} 0.1 μg/mL, 0.2 μM). Compound **17** also showed moderate activity against *Mycobacterium intracellulare*, with an IC_{50} value of 10 μg/mL (29 μM) (ciproflaxin, IC_{50} 0.4 μg/mL, 1 μM), and compound **16** showed weak activity against *Cryptococcus neoformans* with an IC_{50} value of 20 μg/mL (63 μM) (amphotericin B, IC_{50} 0.75 μg/mL, 0.8 μM).

The results indicate that an increase in the number of double bonds in the side chain increases anti-MRSA activity and that the change of isolated *cis-cis* double bonds system to conjugated *trans-cis* moieties decreases the antimicrobial activity.

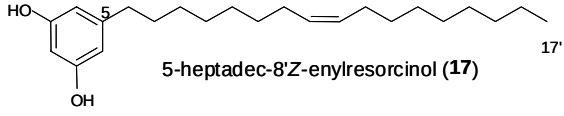
In the screening of basidiomycete cultures from Chile for the production of antimicrobial metabolites, the resupinate polypore *Serpula himantoides* (Coniophoraceae) was identified as species inhibiting growth of bacteria and fungi (Aqueveque et al., 2002). Mycelial cultures of an active strain were derived from the spore print of a fruiting body. Fermentation was



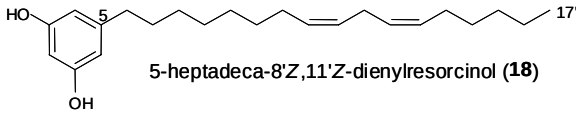
5-pentadec-8'*Z*-enylresorcinol (**15**)



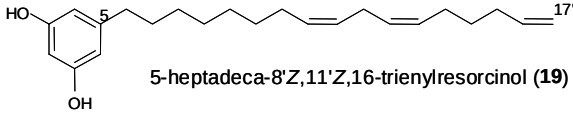
5-pentadeca-8'*Z*,11'*Z*-dienylresorcinol (**16**)



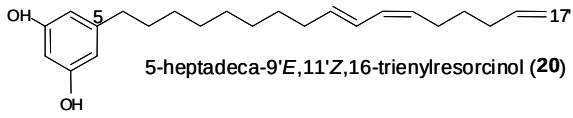
5-heptadec-8'*Z*-enylresorcinol (**17**)



5-heptadeca-8'*Z*,11'*Z*-dienylresorcinol (**18**)

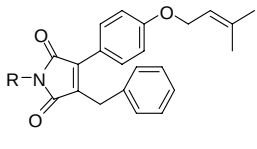


5-heptadeca-8'*Z*,11'*Z*,16-trienylresorcinol (**19**)

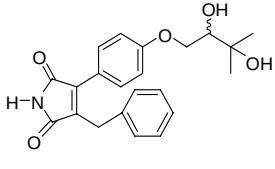


5-heptadeca-9'*E*,11'*Z*,16-trienylresorcinol (**20**)

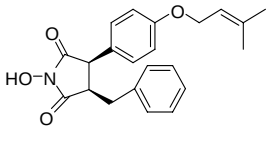
carried out in YMG medium at 24ºC for 240 hours when the antibacterial activity against *Bacillus brevis* and *B. subtilis* of the extract had reached its maximum. Bioactivity-guided isolation provided four active metabolites, himanimides A-D (**21**-**24**). Himanimide C (**23**) exhibited the strongest effects of all four compounds. It showed fungicidal effects against *Alternaria porri*, *Aspergillus ochraceus* and *Phytium irregulare* with MIC values of 25 µg/mL (69 µM) and fungistatic effects against *Absidia glauca*, *Cladosporium cladosporiodes*, *Curvluaria lunata*, *Zygorhynchus moelleri*, *Nadsonia fulvescens* and *Saccharomyces cerevisiae* with the MIC values of 10 µg/mL (28 µM).



himanimide A (**21**) R=H
himanimide C (**23**) R=OH



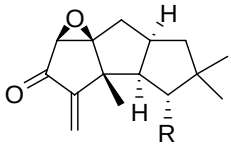
himanimide B (**22**)



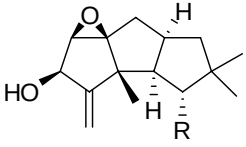
himanimide D (**24**)

It also showed weak to moderate antibacterial activity against Gram-positive bacteria and yeast. No activity was observed against Gram-negative bacteria. It is interesting to note the difference in activity between himanimide C (**23**) and the other three metabolites (**21**,**22** and **24**), which is possibly due to the presence of the double bond and the hydroxylated imine functional group in the maleinimide part of the molecule.

Antimicrobial sesquiterpenes, desoxyhypnophilin (**25**), hypnophilin (**26**), 6,7-epoxy-4(15)-hirsutene-5-ol (**27**), and 6,7-epoxy-4(15)-hirsutene-1,5-diol (**28**) with a hirsutane skeleton were isolated from the wood-decaying polypore, *Lentinus crinitus* (Abate and Abraham, 1994).

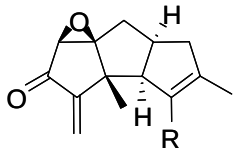


desoxyhypnophilin (**25**) R=H
hypnophilin (**26**) R=OH



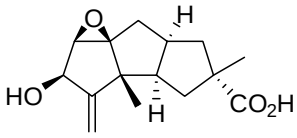
6,7-epoxy-4(15)-hirsutene-5-ol (**27**) R=H
6,7-epoxy-4(15)-hirsutene-1,5-diol (**28**) R=OH

Desoxyhypnophilin (**25**) and hypnophilin (**26**) are active against the Gram-positive bacterium *Bacillus cereus,* and spores of *Aspergillus niger*, *A. flavus,* and *Mucor rouxii* with MIC values of 1-5 µg/mL (4-20 µM). The α,β-unsaturated exomethylene ketone system, present in compounds **25** and **26**, is responsible for antimicrobial activity. Reduction of the carbonyl group in **25** leads to a significant drop in antimicrobial activity of **27** (Abate and Abraham, 1994). Hypnophilin (**26**) together with pleurotellol (**29**) and pleurotellic acid (**30**) were also isolated from fermentation of the gilled mushroom, *Pleurotellus hypnophilus* (Agaricaceae) (Kupka et al., 1981). Hypnophilin (**26**) and pleurotellol (**29**) act in addition as plant growth inhibitors (Lorenzen and Anke, 1998).

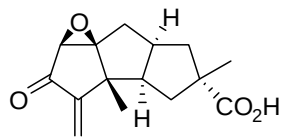


pleurotellol (**29**) R=CH_2OH
pleurotellic acid (**30**) R=CO_2H

Two other hirsutane derivatives, hirsutic acid (**31**) and complicatic acid (**32**), were isolated from the wood-decaying polypore, *Stereum complicatum* (Mellows et al., 1973). Similar to other hirsutanes with an α,β-unsaturated exomethylene ketone system, complicatic acid (**32**) showed moderate antimicrobial activity against *Staphylococcus aureus* (Mantle and Mellows, 1973).

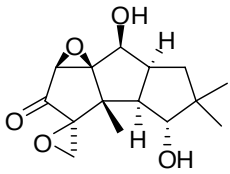


hirsutic acid (**31**)

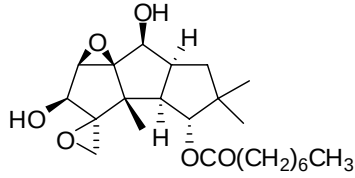


complicatic acid (**32**)

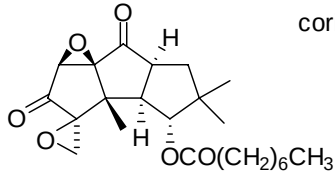
Another antimicrobial hirsutane sesquiterpene, coriolin (**33**), was isolated from the white-rot basidiomycete, *Coriolus consors* (Takeuchi et al., 1969). Coriolin is active against *S. aureus*, *Micrococcus flavus*, *Bacillus subtilis*, and *B. anthracis* with the same MIC values of 12.5 μg/mL (45 μM). A closely related compound isolated from the same fungus, coriolin B (**34**), did not show any antimicrobial activity, but its synthetic derivative, diketocoriolin B (**35**) obtained by oxidation of coriolin B (**34**) showed antimicrobial activity similar to that of coriolin (**33**) (Takeuchi et al., 1971).



coriolin (**33**)

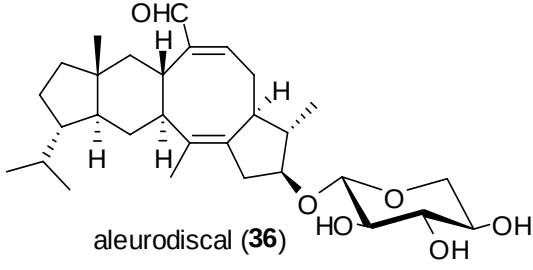


coriolin B (**34**)



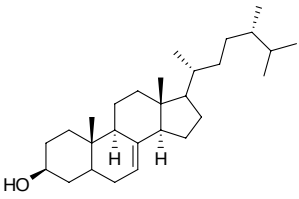
diketocoriolin B (**35**)

A potent antifungal sesterterpene β-D-xyloside, aleurodiscal (**36**), was isolated from another wood-rotting polypore *Aleurodiscus mirabilis* (Lauer et al., 1989). Aleurodiscal (**36**) is selectively active against zygomycetes, especially against *Mucor miehei*.

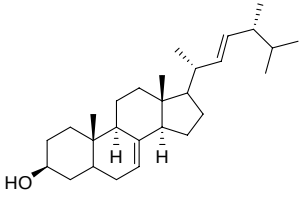


aleurodiscal (**36**)

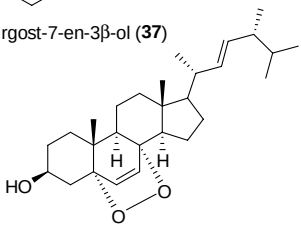
Ganoderma australe, and *G. applanatum*, known commonly as the artist's conk, provide sterols, 5α-ergost-7-en-3β-ol (**37**), 5α-ergost-7,22-dien-3β-ol (**38**), 5,8-epidioxy-5α,8α-ergost-6,22-dien-3β-ol (**39**), and a novel lanostanoid (**40**) that are active predominantly against Gram-positive bacteria (Smania Jr. et al., 1999; Gerber et al., 2000).



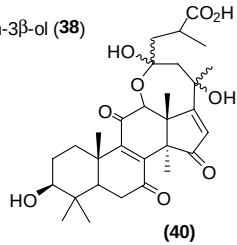
5α-ergost-7-en-3β-ol (**37**)



5α-ergost-7,22-dien-3β-ol (**38**)

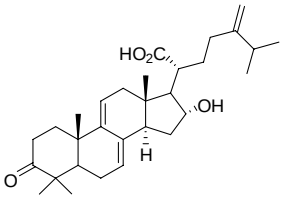


5,8-epidioxy-5α,8α-ergost-6,22-dien-3β-ol (**39**)

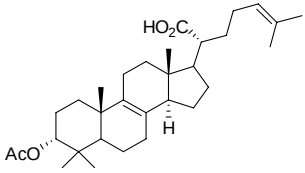


(40)

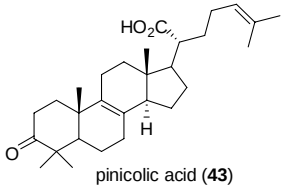
Several lanostanoid derivatives, polyporenic acid C (**41**), 3α-acetyloxylanosta-8,24-dien-21-oic acid (**42**), pinicolic acid A (**43**), trametenolic acid B (**44**), and fomitopsic acid (**45**) isolated from the polypore, *Fomitopsis pinicola* have shown antimicrobial activity against *Bacillus subtilis* in a TLC-bioautography assay in quantities from 0.01 to 1 μg, but did not



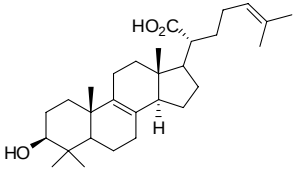
polyporenic acid (**41**)



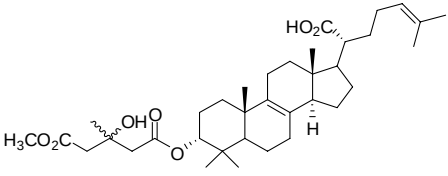
3α-acetyloxylanosta-8,24-dien-21-oic acid (**42**)



pinicolic acid (**43**)



trametenolic acid B (**44**)

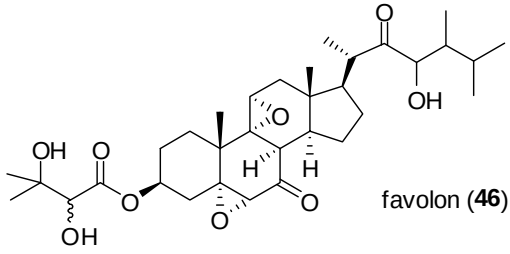


fomitopsic acid (**45**)

inhibit *B. subtilis* in a classic agar dilution assay at concentrations up to 50 µg/mL (Keller et al., 1996).

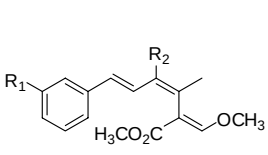
Polyporenic acid C (**41**) was also isolated from *Polyporus benzoinus* and *Piptoporus betulinus* (as *Polyporus betulinus* or *Ungulina betulina*) together with a weaker antibacterial metabolite of undefined structure, ungulinic acid (Locquin et al., 1948; Birkinshaw et al., 1952; Marcus, 1952; Kawagishi et al., 2002; Kamo et al., 2003). Ungulinic acid has 1/6 of the activity of penicillin and tyrothricin but the same as streptomycin, but is inactive against fungi and aerobic bacteria (Locquin et al., 1948; Birkinshaw et al., 1952; Marcus, 1952).

A culture of an Ethiopian *Favolaschia* species produced favolon (**46**), an unusual ergosterone with a B/C-*cis* ring junction. This compound displayed strong antifungal activity against numerous fungal pathogens, with the strongest inhibitions in the agar diffusion assay for *Mucor miehei*, *Paecilomyces varioti*, and *Penicillium islandicum* (Anke et al., 1995).

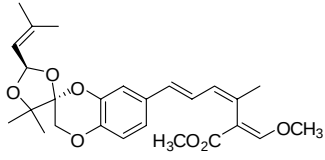


favolon (**46**)

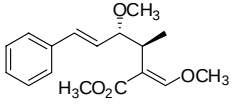
Basidiocarps of the genera *Agaricus*, *Favolaschia,* and *Filoboletus* produced strobilurins A (**47**), E (**48**), F1 (**49**), 9-methoxystrobilurin A (**50**) and oudemansin A (**51**), aromatic antifungal compounds derived by way of the shikimic acid pathway. These compounds exhibited potent antifungal activity (MIC values of 0.1-1 µg/mL, 0.2-3 µM), but they had no discernible antibacterial properties.



strobilurin A (**47**) R_1=H, R_2=H
strobilurin F1 (**49**) R_1=OH, R_2=H
9-methoxystrobilurin A (**50**) R_1=H, R_2=OCH_3



strobilurin E (**48**)

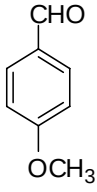


oudemansin A (**51**)

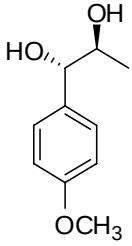
The strobilurins and oudemansins also inhibited the growth of a number of fungal plant pathogens at very low concentrations (Lorenzen and Anke, 1998). They have a unique mode of action, selectively inhibiting

the respiration of fungi by interfering with the ubiquinol oxidation center of the mitochondrial bc1 complex (Lorenzen and Anke, 1998). These compounds have served as natural product prototypes for the design and development of synthetic analogs. Their lack of mammalian toxicity has made them good lead compounds for the development of commercial agricultural fungicides (Clough, 1993).

Very simple aromatic compounds, such as anisaldehyde (**52**) and (4-methoxyphenyl)-1,2-propanediol (**53**), showing weak antifungal activity, were isolated from *Pleurotus pulmonarius* and *Bjerkandera adusta* (DeJong et al., 1994).

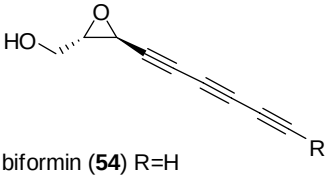


anisaldehyde (**52**)

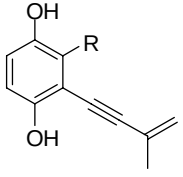


(4-methoxyphenyl)-1,2-propanediol (**53**)

One of the first antimicrobial compounds ever isolated from a polypore was biformin (**54**), a polyacetylenic carbinol. Biformin (**54**) is produced by *Trichaptum biforme* (as *Polyporus biformis*), and is active against a wide variety of bacteria and fungi (Robbins et al., 1947).



biformin (**54**) R=H
trans-2,3-epoxydeca-4,6,8-triyn-1-ol (**55**) R=CH_3

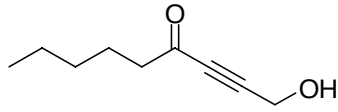


frustulosin (**56**) R=CHO
frustulosinol (**57**) R=CH_2OH

A methyl homolog of biformin (**54**), *trans*-2,3-epoxydeca-4,6,8-triyn-1-ol (**55**), was isolated from the liquid culture of the Ethiopian polypore, *Trametes pubescens* (Dagne et al., 1994). In contrast to biformin, compound **55** did not show antibacterial activity but exhibited antifungal activity against *Aspergillus niger, A. ochraceus, Rhodotorula glutinis, Candida tropicalis*, and *C. albicans* with MIC values of 1, 1, 10, 50, and 50 µg/mL (6, 6, 62, 312, 312 µM), respectively. The aromatic acetylene derivatives, frustulosin (**56**) and frustulosinol (**57**), isolated from the liquid cultures of *Stereum frustulosum* were active against several bacteria such as *Staphylococcus*

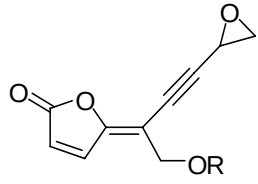
aureus, *Bacillus mycoides*, *B. subtilis*, and also moderately active against *Vibrio cholera* and *V. cholera* phage (Nair and Anchel, 1975; Nair and Anchel, 1977).

Another example of an acetylenic compound exhibiting antifungal activity is the 1-hydroxy-2-nonyn-4-one (**58**) isolated from the fermentation of polypore *Ischnoderma benzoinum* (Anke et al., 1982).



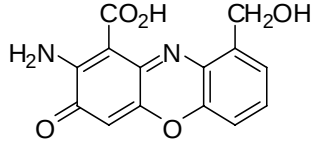
1-hydroxy-2-nonyn-4-one (**58**)

A liquid culture of the strain of the polypore mushroom *Aporpium caryae* (Schweinitz) Teixeira & D.P. Rogers provided a series of furanones with the side chain containing an acetylenic unit (aporpinones). Aporpinone B (**59**) and acetylaporpinone B (**60**) showed weak to moderate antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Levy et al., 2003).



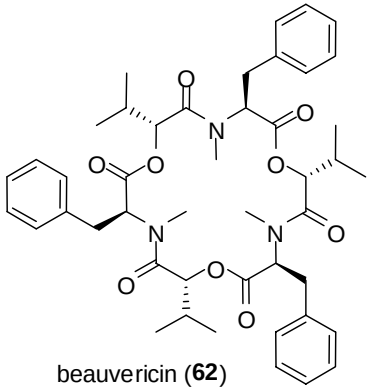
aporpinone B (**59**) R=H
acetylaporpinone B (**60**) R=$COCH_3$

The red polypore, *Pycnoporus sanguineus,* produces cinnabarin (**61**), a phenoxazinone with antimicrobial activity. *Bacillus cereus* and *Leuconostoc plantarum* were the most sensitive to cinnabarin, each being inhibited with an MIC value of 62.5 μg/mL (218 μM) (Smania et al., 1997; Smania et al., 1998).



cinnabarin (**61**)

A large group of antimicrobial secondary metabolites isolated from polypores also includes a cyclodepsipeptide, beauvericin (**62**). Beauvericin (**62**) is produced by the bright yellow polypore *Laetiporus sulphureus* (as *Polyporus sulphureus*), commonly known as 'Chicken-of-the-Woods' (Deol et al., 1978). Beauvericin is also considered as a mycotoxin produced by hypocrealean ascomycetes in grain (Morrison et al., 2002).



beauvericin (**62**)

An antifungal polypeptide, alveolarin with molecular weight of 28kDa was isolated from fresh fruiting bodies of *Polyporus alveolaris* (Wang et al., 2004). Alveolarin inhibited mycelial growth of *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*. Another antifungal polypeptide, pleurostrin (MW = 7 kDa) with antifungal activity against the same species was isolated from fresh fruiting bodies of oyster mushroom (*Pleurotus ostreatus*) (Chu et al., 2005). Recently, a 15kDa antifungal protein, ganodermin, was isolated from the very well known polypore *Ganoderma lucidum* (Wang and Ng, 2006). Ganodermin inhibited the mycelial growth of *Botrytis cinerea*, *Fusarium oxysporum*, and *Physalospora piricola* with an IC_{50} value of 15.2, 12.4, and 18.1 μM, respectively.

A number of polypores exhibit immunoprotective activity and provide protection against a variety of infectious diseases. This kind of activity is associated mainly with the presence of polysaccharides. PSK, a protein-bound polysaccharide isolated from *Trametes versicolor* (as *Coriolus versicolor*), was found to increase resistance in mice against infection with *Listeria monocytogenes* by enhancing oxygen metabolism of the host macrophages (Saito et al., 1988).

Antiviral metabolites

In their excellent review article on mushroom antivirals, Brandt and Piraino divided the antiviral compounds from fungi into two major classes: (i) those

that act indirectly as biological response modifiers (usually from polysaccharide fractions); and (ii), those that act directly as viral inhibitors (Brandt and Piraino, 2000; Piraino, 2006). In polypores, however, several polysaccharide fractions display direct inhibitory effects on various viruses. The polysaccharide preparation, PSK from *Trametes versicolor,* commonly known as turkey tail, was found to have an antiviral effect on human immunodeficiency virus (HIV) *in vitro* (Tochikura et al., 1987; Tochikura et al., 1989). One of the mechanisms of this effect was due to inhibition of the binding of HIV with lymphocytes. PSK inhibited reverse transcriptase of avian myeloblastosis virus *in vitro* (Hirose et al., 1987). PSK was also shown to provide protection against exogenous and endogenous infections by the murine cytomegalovirus (MCMV) (Okada and Minamishima, 1987).

In contrast to PSK, another protein-bound polysaccharide, PSP, isolated from *Trametes versicolor* is not a 'true' antiviral and acts indirectly by immunostimulation (Brandt and Piraino, 2000). PSP was reported to inhibit the binding of HIV-1 gp120 to immobilized CD4 receptor with an IC_{50} value of 150 μg/mL, and recombinant HIV-1 reverse transcriptase with an IC_{50} value of 6.25 μg/mL (Colins and Ng, 1997). Both the polysaccharides PSK and PSP are heteroglucans with α-(1→4) and β-(1→3)glucosidic linkages, with a protein or polypeptide component. The presence of fucose in PSK, and rhamnose and arabinose in PSP, distinguishes the two protein-bound polysaccharides, which are otherwise chemically similar (Ng, 1998).

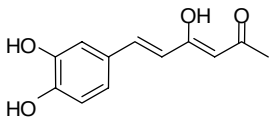
An extract of the culture medium of mycelia of very well known polypore mushroom Shiitake (*Lentinus edodes*) showed activity against HIV *in vitro* (Tochikura et al., 1988). The spore extract of the same mushroom inhibited the A/SW-15 influenza virus infection in mice after the single i.p. injection by inducing an interferon (Suzuki et al., 1976).

Aqueous extracts from four polypores, *Fomitella supina, Phellinus rhabarbarinus, Trichaptum perrottotti*, and *Trametes cubensis* showed strong anti-HIV-1 activity without toxicity toward lymphocytic cells. It was demonstrated that the active compounds of these extracts act by a mechanism of direct virion inactivation and inhibition of syncytium formation in an *in vitro* culture system (Walder et al., 1995).

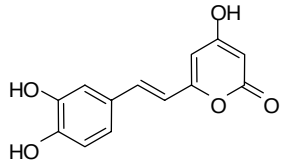
Water-soluble preparations from carpophores of *Ganoderma applanatum* (as *Elfvingia applanata*) exhibited potent antiviral activity against vesicular stomatitis virus Indiana serotype VSV (IND) (Eo et al., 2001).

Water-soluble lignins isolated from the sclerotia of the polypore *Inonotus obliquus*, commonly known as 'chaga', inhibited HIV protease with an IC_{50} value of 2.5 μg/mL (Ichimura et al., 1998).

Two phenolic compounds, hispolon (**63**) and hispidin (**64**), isolated from the basidiocarps of *Inonotus hispidus* showed considerable antiviral activity against influenza viruses type A and B (Awadh Ali et al., 2003).



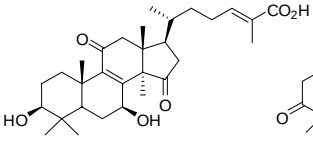
hispolon (**63**)



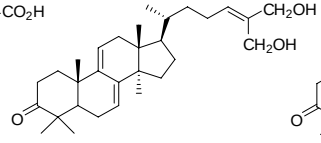
hispidin (**64**)

The filtrate from the culture of the polypore *Fomes fomentarius*, 'tinder conk', is highly active against the mechanical transmission of tobacco mosaic virus (TMV) with an IC_{50} value of 10 μg/mL, and it has similar effects against TMV infection on bell pepper and tomato plants (Aoki et al., 1993).

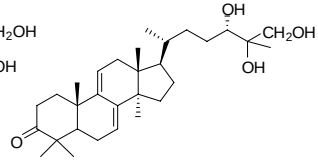
The fruiting bodies of *Ganoderma lucidum* are the source of antiviral triterpenoids. Ganoderic acid β (**65**) isolated from the spores of *G. lucidum* showed significant anti-HIV-1 protease activity with an IC_{50} value of 20 μM (Min et al., 1998). The same species also produced ganoderiol F (**66**) and ganodermanontriol (**67**) that have anti-HIV-1 activity (El-Mekkawy et al., 1998).



ganoderic acid β (**65**)

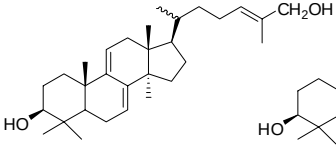


ganoderiol F (**66**)

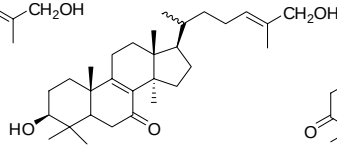


ganodermanontriol (**67**)

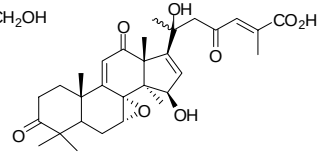
Antiviral triterpenes were also isolated from the European polypore *Ganoderma pfeiferri*. Ganodermadiol (**68**), lucidadiol (**69**), and applanoxidic acid (**70**) showed antiviral activity against influenza virus type A and HSV-1 (Mothana et al., 2003).



ganodermadiol (**68**)



lucidadiol (**69**)



applanoxidic acid (**70**)

Conclusions and future perspectives

Polypore fungi are major sources of biologically active natural products among species of the diverse fungal phylum Basidomycota. They provide

a rich variety of active secondary metabolites and polysaccharides. Certain polypores have repeatedly been found to contain active compounds. These include *Trametes versicolor*, *Laetiporus sulphureus*, and several species of *Ganoderma*, with long-lived fruiting bodies that ideally resist decay during their relatively long periods (weeks to months) of active basidiospore production. This is evident from the large number of compounds isolated from polypores that have proved to have significant antimicrobial activities, making them good candidates for critically needed new antibiotics. Sclerotia, the long-lived underground resistant mycelial structures of polypores such as *Grifola umbellata* and *Wolfiporia cocos,* also are good sources of secondary metabolites. Polysaccharide fractions of many polypores have shown remarkable anticancer effects *in vivo* through potentiation and stimulation of the entire immune system.

While a functional role for antibiotics can be assumed, a role for the compounds in the fungi that synthesize them, however, is unknown. Another neglected area of research in relation to the secondary metabolites of basidiomycetes is the difference in production of different compounds in different life history states, the mycelium (somatic assimilative state) and basidiocarp (reproductive state). They apparently are distinctive not only in function, but also, in production of metabolites.

The emergence of H5N1 avian influenza, SARS and the threat of other viruses in bioterrorism has created an urgent need for identifying new antiviral agents. Polypore mushrooms provide extracts and compounds that are active against a large spectrum of viruses including avian influenza, SARS, West Nile virus, Dengue, yellow fever and other viruses (Stamets, 2006).

Among the biologically active compounds from Basidiomycetes, a number from Aphyllophorales (polypores) have found their way to the market. In Japan, the polysaccharide anticancer drug PSK (Krestin®) isolated from the polypore *Trametes versicolor* (as *Coriolus versicolor*) is on the market, together with two other drugs from non-polyporous wood-decaying fungi: Lentinan (Enzolen™) from *Lentinus edodes* (Shiitake), and Schizophyllan (Sonifilan®) from *Schizophyllum commune*. Several polysaccharide preparations from basidiomycetes, including polypores such as *Grifola frondosa*, *Ganoderma lucidum*, and *Trametes versicolor,* are in clinical trials in the Peoples Republic of China. Extracts from numerous Aphyllophorales are also available all over the world as nutritional supplements or herbal remedies. There is an intense interest in these so-called 'mushroom nutriceuticals' by consumers. The worldwide market value of mushroom dietary supplement products from *Ganoderma lucidum* species is estimated to be US $ 5-6 billion per year, with US $ 1.6 billion for the United States (Chang and Buswell, 1999; Wasser et al., 2000; Chang and Buswell, 2003).

Major research on isolation of pharmacologically active compounds from polypores, as well as other Basidiomycetes, comes from Germany, Japan, Korea, and the People's Republic of China, the countries with the historically best established tradition of the use of medicinal mushrooms. Unfortunately, the United States has been poorly represented in this research field. Considering, however, the leading role of the U.S in the study of natural products worldwide, this gap could soon be filled. The large and well preserved natural resources of North America, with a rich diversity of higher fungi, including polypores, provide a good base for extensive research in isolation and biological evaluation of natural products from mushrooms.

Acknowledgements

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3 Naturally Occurring Anti-*Salmonella* Agents and Their Modes of Action

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1. Introduction

Salmonellosis is one of the most frequently occurring bacterial food-borne illness. The salmonellae are Gram-negative non-spore-forming rods. There are over 2500 serovars of *Salmonella*, all of which are pathogenic for humans. The salmonellae ferment glucose but not lactose or sucrose. The study to search for anti-*Salmonella* agents was initiated by the request to solve the problem of contamination in the fruit of *Piper nigrum* (Piperaceae), commonly known as pepper, in the Amazon basin. On the basis of a preliminary survey, this contamination has been likely caused by increased large-scale poultry farms around the area, since important sources of *Salmonella* contamination for foods are poultry and rodents. For example, chickens may be infected with any number of types of *Salmonella*, which are then found in the fecal matter. In addition, infected rodents, rats, mice and bats may contaminate unprotected pepper with their feces and thus spread *Salmonella* bacteria (Frazier and Westhoff, 1988; Tyrrel and Quinton, 2003). In order to search for anti-*Salmonella* agents, *Salmonella choleraesuis* subsp. *choleraesuis* ATCC 35640 was selected as an example since this bacterium most frequently causes septicemia, although septicemia can be caused by any *Salmonella*. There is a great need for effective antibacterial agents but no appropriate anti-*Salmonella* agent for food is currently available; hence, safe and effective anti-*Salmonella* agents are urgently needed. Phytochemicals characterized from edible plants have the potential of filling this need because their structures are different from those of the well-studied microbial sources; therefore, their modes of action may very likely differ.

In our continuing search for antimicrobial agents from food spices, several phytochemicals previously characterized as antibacterial agents against Gram-negative bacteria were found to exhibit antibacterial activity against *S. choleraesuis*. Among them, volatile compounds such as (2*E*)-alkenals and alkanals were characterized from various edible plants such as the coriander *Coriander sativum* L. (Umbelliferae) (Kubo et al., 2004a), the olive *Olea europaea* L. (Oleaceae) (Kubo et al., 1995a), and the cashew *Anacardium occidentale* (Anacardiaceae) (Muroi et al., 1993) were found to exhibit antibacterial activity against *S. choleraesuis* (Kubo et al., 2004b). In addition, a bicyclic sesquiterpene dialdehyde, polygodial (**1**) (Figure 1) isolated from various food spices and medicinal plants such as *Polygonum hydropiper* and *P. punctatum* (Polygonaceae); *Warburgia ugandensis* and *W. stuhlmannii* (Canaraceae); and *Drymis winteri* and *Tasmania lanceolata* (Winteraceae) (Ban et al., 2000) was described to show moderate antibacterial activity against *S. choleraesuis* (Kubo et al., 2001). The maximum antimicrobial activity of (2*E*)-alkenals is dependent on the balance of the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head) (Kubo et al., 1995b and 2003a). It is well known that the hydrophobicity of molecules is often associated with biological action (Hansch and Dunn, 1972). However, the rationale for this observation, especially the role of the hydrophobic portion, is still poorly understood and widely debated. To clarify this, (2*E*)-alkenals are a superior model for structure and anti-*Salmonella* activity relationship (SAR) study because these molecules possess the same hydrophilic portion, the enal group, which explain the role of the hydrophobic alkyl portion. In addition, a series of (2*E*)-alkenals and their related analogues are common in many plants (Kim et al., 1995; Kubo and Kubo, 1995; Kubo et al., 1995, 1996 and 1999; Kubo and Fujita, 2001) and are readily available. Therefore, a series of aliphatic (2*E*)-alkenals, as well as the corresponding alkanals and alkanols, from C_5 to C_{13} were tested for their antibacterial activity against *S. choleraesuis* to gain new insights into their antibacterial action on a molecular basis (Kubo et al., 2004b). This work has been reported as a result

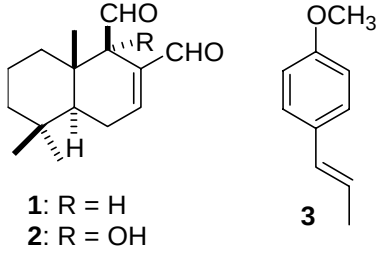

Fig. 1. Structures of polygodial (**1**), warburganal (**2**) and anethole (**3**).

of sporadic research (Kubo et al., 1993a, 1993b, 1995a, 1995b, 1996, 1999, 2003a, 2003b, 2004a and 2004b; Muroi et al., 1993; Kubo and Fujita, 2001), so it is an opportune time to review and include all that is currently known into one chapter.

2. (2*E*)-Alkenals

A homologous series of (2*E*)-alkenals and alkanals were assayed for their antibacterial activity against *S. choleraesuis* using a 2-fold serial broth dilution method. The results are listed in Table 1. As expected, their antibacterial activity against this food-borne bacterium is correlated with the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head). The activity grew with increasing alkyl chain length up to (2*E*)-dodecenal (C_{12}). Thus, (2*E*)-dodecenal is the most effective bactericide against *S. choleraesuis*, followed by (2*E*)-undecenal (C_{11}). It appears that *S. choleraesuis* showed different susceptibilities to aldehydes possessing different chain lengths. This result is broadly similar to those of the corresponding alkanols against many microorganisms (Kubo et al. 1995b and 2003b), indicating, at least in part, the similarity of their mode of action. The range of the antibacterial activity of the (2*E*)-alkenals tested against *S. choleraesuis* is between 6.25 and 200 μg/ml, and the MICs and MBCs are markedly the same. Both the MIC and MBC of the most potent (2*E*)-dodecenal are 6.25 μg/ml (34 μM). Notably, this MBC value is slightly more potent than that of gentamicin (Table 2).

Table 1. Antibacterial activity (μg/ml) of aldehydes against *S. choleraesuis* subsp. *choleraesuis* ATCC 35640

Aldehydes tested	*MIC (MBC)*	
	(2E)-Alkenal	*Alkanal*
C_5	200 (*200*)	>800 (–)
C_6	100 (*100*)	400 (*800*)
C_7	100 (*100*)	400 (*400*)
C_8	100 (*100*)	200 (*400*)
C_9	25 (*25*)	100 (*200*)
C_{10}	50 (*50*)	100 (*100*)
C_{11}	12.5 (*12.5*)	100 (*100*)
C_{12}	6.25 (*6.25*)	100 (*100*)
C_{13}	25 (*200*)	>800 (–)

Numbers in *italic* type in parenthesis are *MBC.*
(–) Not tested.

Table 2. Antibacterial activity (μg/ml) of the C_6 compounds and gentamycin against *S. choleraesuis* ATCC 35640

Compounds tested	*MIC (MBC)*
(2*E*)-Hexenal	100 (*100*)
Hexanal	400 (*800*)
Hexanol	>1600 (>*1600*)
Hexanoic acid	400 (*400*)
Gentamicin	12.5 (*12.5*)

Numbers in *italic* type in parenthesis are *MBC.*

The bactericidal effect of (2*E*)-dodecenal was confirmed by the time kill curve method as shown in Figure 2. Cultures of *S. choleraesuis*, with a cell density of 1×10^5 CFU/ml, were exposed to three different concentrations of (2*E*)-dodecenal. The number of viable cells was determined following different periods of incubation with (2*E*)-dodecenal. The result verifies that the MIC and MBC are the same. It shows that ½MIC slowed growth, but the final cell count was not significantly different from the control. Notably, lethality occurred quickly, within the first 1 h after the addition of (2*E*)-dodecenal. This rapid lethality very likely indicates that antibacterial activity of (2*E*)-dodecenal against *S. choleraesuis* is associated with the

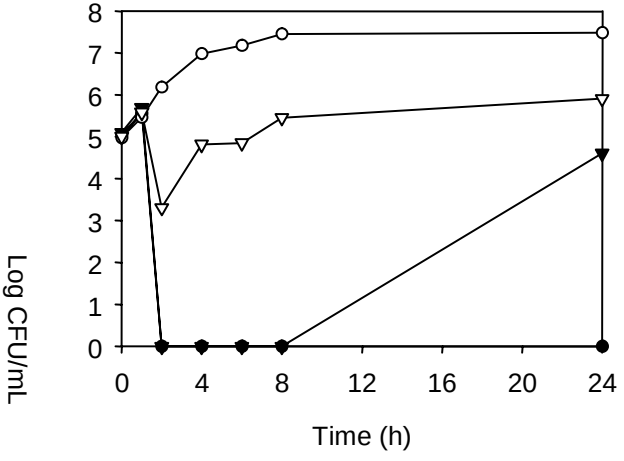

Fig. 2. Effect of (2*E*)-dodecenal on the growth of *S. choleraesuis* subsp. *choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C with 0 (○); 1.56 (▽); 3.13 (▼); and 6.25 (●) μg/ml of (2*E*)-dodecenal. The drug was added after 1-h incubation. Viability was established by the number of colonies formed on NYG plate after incubation at 30°C in for 24 h.

disruption of the membrane (Trombetta et al., 2002), similar to its effects found against *Saccharomyces cerevisiae*.

The bactericidal effect of (2*E*)-hexenal was also confirmed by the time kill curve experiment as shown in Figure 3. Cultures of *S. choleraesuis*, with a cell density of 1×10^5 CFU/ml, were exposed to two different concentrations of (2*E*)-hexenal. The number of viable cells was determined following different periods of incubation with (2*E*)-hexenal. The result verifies that MIC and MBC are the same. Lethality occurred slower than that of (2*E*)-dodecenal. The result obtained indicates that the mode of antibacterial activity of (2*E*)-hexenal and (2*E*)-dodecenal against *S. choleraesuis* differs to some extent.

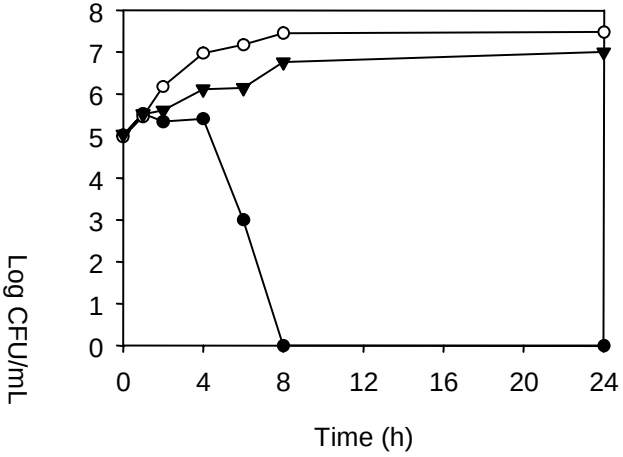

Fig. 3. Effect of (2*E*)-hexenal on the growth of *S. choleraesuis* subsp. *choleraesuis*. Exponentially growing cells were inoculated in NYG broth and then cultured at 37°C with 0 (○); 50 (▼), or 100 (●) μg/ml of (2*E*)-hexenal. The drug was added after 1-h incubation. Viability was established by the number of colonies formed on NYG plate after incubation at 30°C in for 24 h.

The bactericidal effect of (2*E*)-hexenal (C_6) needed 7 h. Such a slow cell death is thought to proceed independent of the membrane disruptive action. The effects of (2*E*)-dodecenal and (2*E*)-hexenal against *S. choleraesuis* were further tested by holding the viable cell number in the presence of chloramphenicol. This antibiotic is known to restrict cell division by inhibiting protein synthesis. Figure 4 shows that the effect of chloramphenicol against *S. choleraesuis* cells is bacteriostatic for the first 3 h after the addition of the drug. It should be noted that chloramphenicol is known to be bacteriostatic for a wide range of Gram-positive and Gram-negative bacteria, but this antibiotic expressed a bactericidal effect against *S.*

choleraesuis after 8-h incubation. In the presence of chloramphenicol, (2*E*)-hexenal decreased viable cell numbers slightly more quickly than in its absence. (2*E*)-dodecenal induced rapid decrease in viability regardless of the presence of chloramphenicol. The inhibition of cell division by chloramphenicol did not influence the bactericidal effects of (2*E*)-hexenal and (2*E*)-dodecenal. The reduced viability might not be due to interaction with the biosynthesis of cell wall or plasma membrane components. The

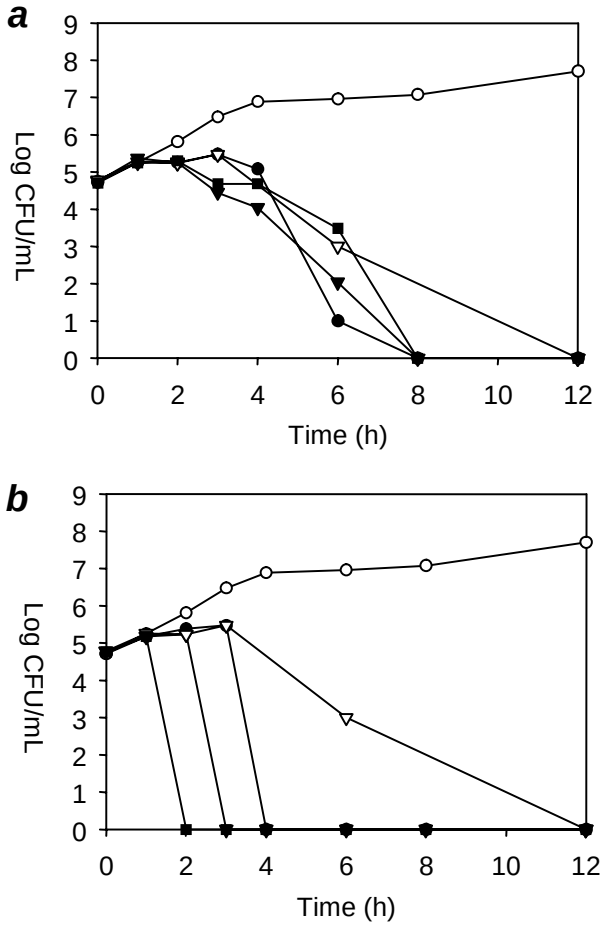

Fig. 4. Effect of (***a***) (2*E*)-hexenal and (***b***) (2*E*)-dodecenal in the presence of chloramphenicol against *S. choleraesuis* subsp. *choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. Chloramphenicol 0 (○); and 6.25 (▽, ▼, ●) μg/ml, was added to the culture after 1-h cultivation. (a) (2*E*)-Hexenal (100 μg/ml) or (b) (2*E*)-Dodecenal (6.25 μg/ml) were added at 1 (■), 2 (▼), and 3 (●) h.

synthesis of macromolecules such as DNA, RNA and proteins was not related to the reduction. The observation that the rapid bactericidal effect of (2*E*)-dodecenal very likely indicates that the primary target of (2*E*)-dodecenal is on the cell membrane.

Subsequently, hexanal (C_6) was also found to exhibit the antibacterial activity against *S. choleraesuis* with MIC and MBC of 400 and 800 µg/ml, respectively. It appears that the antibacterial activity against *S. choleraesuis* should not be specific to (2*E*)-alkenals because the conjugated double bond is not essential in eliciting activity, but is involved with increasing the activity. This prompted us to assay the corresponding alkanals for their antibacterial activity against *S. choleraesuis* for comparison. The results are listed in Table 1. The activity of alkanals is weaker than those of the corresponding (2*E*)-alkenals. Similar to (2*E*)-alkenals, their MIC and MBC values are approximately the same and the activity also increased in general with increasing carbon chain length up to dodecanal (C_{12}). It should be noted, however, that there is a slight difference between (2*E*)-alkenals and alkanals. For example, decanal (C_{10}), undecanal (C_{11}), and dodecanal are the most effective but their MIC and MBC values against *S. choleraesuis* are all the same. The increase in the activity as carbon-chain length increases is not distinct in the case of alkanals as compared to those of (2*E*)-alkenals. The bactericidal effect of hexanal and dodecanal were also confirmed by the time kill curve method (data not shown).

The activity often disappears after the chain length reached the maximum activity and this phenomenon is known as the cutoff. As expected, dodecanal (C_{12}) was the most effective against *S. choleraesuis* with both MIC and MBC of 100 µg/ml, while tridecanal (C_{13}) did not show any activity up to 800 µg/ml. Noticeably, this cutoff was not observed with the (2*E*)-alkenal series against *S. choleraesuis*. That is, (2*E*)-tridecenal exhibits some activity, though to a lesser extent than (2*E*)-dodecenal. This difference in susceptibility of *S. choleraesuis* to (2*E*)-alkenals possessing different chain lengths still remains largely unclear. Since the hydrophobic forces are more favorable than hydrogen bonding forces, this may help to explain the cutoff in that the compound is pulled further into membrane and loses the orientation required for bilayer disruption. The hydrophilic aldehyde group first binds with an intermolecular hydrogen bond like a 'hook' by attaching itself to the hydrophilic portion of the membrane surface, at which point the hydrophobic alkyl portion of the molecule is able to enter into the membrane lipid bilayers (Kubo et al., 1995a and 2003a).

In the current study, (2*E*)-hexenal was found to be effective against *Pseudomonas aeruginosa* with an MBC of 800 µg/ml. This troublesome bacterium is the most resistant organism to phytochemicals, followed by *Escherichia coli* and *Enterobacter aerogenes* (Kubo et al., 1996). The activity of

(2*E*)-alkenals against *P. aeruginosa* decreased with an increasing carbon chain length. (2*E*)-Decenal did not exhibit any activity against *P. aeruginosa* up to 1600 μg/ml. The different susceptibilities between *S. choleraesuis* and *P. aeruginosa* may be caused by their different permeabilities of the outer membrane layer since this plays a major role in the general resistance of Gram-negative bacteria especially to lipophilic antibiotics. The outer membrane is known to surround most Gram-negative bacteria and this function as an effective but less specific barrier (Nikaido, 1994). It is logical to assume that most of the lipophilic (2*E*)-alkenal molecules being dissolved in the medium are incorporated into the outer membrane and hence hardly reach the plasma membrane of *P. aeruginosa*. Notably, (2*E*)-hexenal was the only active compound against all of the Gram-negative bacteria tested. Thus, the other (2*E*)-alkenals as well as alkanals tested did not show any activity against *E. coli*, *P. aeruginosa*, *E. aerogenes*, and *Proteus vulgaris* up to 800 μg/ml. In our continuing search for antimicrobial agents from plants, a number of active principles have been characterized. However, only a few of them showed activity against Gram-negative bacteria, especially the *Pseudomonas* species. (2*E*)-hexenal is one of the rare phytochemicals found as antibacterial agents against *P. aeruginosa*. The bactericidal effect of (2*E*)-dodecenal against *S. choleraesuis* occurred faster than that of (2*E*)-hexenal (Kubo and Fujita, 2001). The phenomenon observed very likely indicates that primary action of (2*E*)-dodecenal is on the cell membrane.

We first characterized (2*E*)-hexenal as the principal antimicrobial agent from the cashew apple (Muroi et al. 1993) and subsequently olive oil (Kubo et al., 1995a). Soon after, we became aware that this common α,β-unsaturated aldehyde known as 'leaf aldehyde' is widely distributed in many plants (Hatanaka, 1993). (2*E*)-hexenal may be a key defense chemical (postinhibitin) in plants against microbial attacks. Safety is a primary consideration for anti-*Salmonella* agents, especially those in food products, which may be utilized in unregulated quantities on a regular basis. The phytochemicals characterized as anti-*Salmonella* agents isolated from plants being used as food spices and/or characterized, as flavor substances in many edible plants should be superior as compared to a nonnatural one.

The antibacterial activity of (2*E*)-alkenals is nonspecific, and the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group up to (2*E*)-dodecenal. In the time kill experiment of (2*E*)-dodecenal against *S. choleraesuis*, (i) lethality occurred notably quickly, within the first 1 h after the addition of (2*E*)-dodecenal, (ii) the bactericidal activity was found at any growth stage, and (iii) (2*E*)-dodecenal rapidly killed *S. choleraesuis* cells in which cell division was inhibited by chloramphenicol. Taking together this study and our previous report (Kubo et al., 1995a), the antibacterial activity of (2*E*)-dodecenal against *S.*

choleraesuis is mediated primarily due to its nonionic surfactant property, although it cannot be inferred that membrane damage is the only cause of the lethal effect.

The greater bactericidal activity of (2*E*)-dodecenal than that of (2*E*)-hexenal against *S. choleraesuis* is due primarily to a balance between the hydrophilicity of the unsaturated aldehyde subunit and the hydrophobicity of the alkyl portion of the molecule similar to their action against *S. cerevisiae* (Kubo et al., 2003a). The amount of (2*E*)-alkenals entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. The short chain (2*E*)-alkenals enter the cell by passive diffusion across the plasma membrane and/or through porin channels. The more lipophilic long-chain (2*E*)-alkenals molecules being dissolved in the medium are incorporated into the lipid bilayers, similar to those found for alkanols (Kubo et al., 1995b and 2003b). In contrast to alkanols, α,β-unsaturated aldehydes are chemically highly reactive substances and they readily react with biologically important nucleophilic groups, such as sulfhydryl, amino, or hydroxyl. The main reaction appears to be 1,4-addition under physiological conditions, although the formation of Schiff bases is also possible (Schauenstein et al., 1977). Once inside the cells, (2*E*)-alkenals may react with various intercellular components. For instance, bacteria are known to protect themselves against reactive oxygen species in various ways, and some of the most ubiquitous systems include glutathione (Brul and Coote, 1999). It appears that (2*E*)-dodecenal mainly acts as a surfactant and then inhibits various cellular functions in an ordered sequential mechanism, while (2*E*)-hexenal behaves reversely. In our previous experiment that (2*E*)-undecenal rapidly adsorbed onto the surface of *S. cerevisiae* cells but (2*E*)-hexenal did slightly (Kubo et al., 2003a). It appears that *S. cerevisiae* showed different affinities to (2*E*)-alkenal having different alkyl chain lengths (Kubo et al., 2003b), and this may support the aforementioned postulate.

The same series of (2*E*)-alkenals has been reported to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver, and the potency grew with increasing chain length up to (2*E*)-undecenal, similar to those found for alkanols (Hammond and Kubo, 2000). On the other hand, (2*E*)-alkenals did not inhibit the bacterial membrane respiratory system (Haraguchi et al., 1992). For example, neither (2*E*)-hexenal nor (2*E*)-undecenal inhibited NADH oxidase prepared from the *P. aeruginosa* IFO 3080 cells up to 100 μM.

In our previous articles on structure-antifungal activity relationship (SAR) studies with the same series of acyclic (2*E*)-alkenals, we reported that the antifungal activity of amphipathic medium chain (C_9-C_{12}) (2*E*)-alkenals against *S. cerevisiae* was mediated. This was largely due to their

nonionic surface-active properties and hence, the maximum activity can be obtained when balance between hydrophilic and hydrophobic portions becomes the most appropriate, similar to being described for acyclic alkanols (Kubo et al., 1995b and 2003b). In other words, the antifungal activity of (2*E*)-alkenals against *S. cerevisiae* is due mainly to biophysical process. This concept can be extended to the antibacterial activity of the same medium chain (2*E*)-alkenals against *S. choleraesuis*. Moreover, the antimicrobial activity of (2*E*)-alkenals is non-specific and the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group, up to (2*E*)-dodecenal. The results observed support medium chain (2*E*)-alkenals' ability to function at least in part as nonionic surfactants.

The common nature among these aldehydes should be considered in that the electron negativity on the aldehyde oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of particular hydrophilic aldehyde group. They could enter the molecular structure of the membrane with the polar aldehyde group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase by dispersion forces. Eventually, when the dispersion force becomes greater than the hydrogen bonding force, the balance is destroyed and the activity disappears. In connection with this, the hydrophobic bonding energy between an average fatty acid ester and a completely hydrophobic peptide is approximately 12 kcal/mol. Addition of a hydrogen bond between a peptide and a fatty ester's carbonyl adds another 3-6 kcal/mol. Furthermore, aldehydes first approach the binding site with the electron negativity of the aldehyde oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayer.

Given the surfactant-like properties of medium-chain (2*E*)-alkenals, it is possible to suggest that (2*E*)-alkenals also act at the lipid-protein interface of integral proteins, such as ion channels and/or transport proteins, denaturing their functional conformation in a similar manner found for alkanols (Kubo et al., 1995b and 2003b). Thus, the amphipathic medium-chain (2*E*)-alkenals disrupt the hydrogen bonding in the lipid-protein interface in *S. choleraesuis*. The data obtained are consistent with an effect on the bulk membrane rather than a direct interaction of the specific target proteins, and (2*E*)-alkenals' non-specificity of antimicrobial activity supports this assumption. The possibility of the anti-*Salmonella* activity of the medium-chain (2*E*)-alkenals is due to their nonionic surfactant property, but this may not be the case for short-chain ($< C_9$) (2*E*)-alkenals.

The short chain (2*E*)-alkenals enter into the cell by passive diffusion across the plasma membrane and/or through porin channels (Schulz, 1996). Once inside the cell, their α,β-unsaturated aldehyde (enal) moiety is chemically highly reactive and hence, they may readily react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl (Schauenstein et al., 1977). Sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell. Bacteria protect themselves against hydrogen peroxide in various ways (Brul and Coote, 1999), and some of the most ubiquitous systems include glutathione. (2*E*)-alkenals causes depletion of cytoplasmic and mitochondrial glutathione which functions in eliminating reactive oxygen species, similar to found for polygodial (Machida et al., 1999). This (2*E*)-alkenal mediated depletion of intercellular glutathione can be explained by a direct interaction between the enal moiety and the sulfhydryl group of glutathione by a Michael-type addition. This may reveal the reason why (2*E*)-alkenals exhibit in general more potent and broader antimicrobial activity than those of the corresponding alkanals and alkanols. In the case against *S. choleraesuis*, (2*E*)-hexenal exhibited the bactericidal activity against this food borne bacterium with an MBC of 100 μg/ml, whereas hexanol did not show any activity up to 1600 μg/ml.

Moreover, the leakage of carboxyfluorescein (CF) in liposomes of phosphatidylcholine (PC) following exposure to (2*E*)-alkenals was previously reported (Trombetta et al., 2002). Interestingly, (2*E*)-alkenals caused rapid CF leakage from PC liposomes and the effectiveness order correlated well with the alkyl chain length. Thus, (2*E*)-nonenal was more effective in inducing CF leakage from PC liposomes than that of (2*E*)-hexenal. This previous report also supports the surfactant concept.

The process by which antibacterial agents reach the action sites in living bacteria is usually neglected in the cell-free experiment, but this must be taken into account in the current study. The inner and outer surfaces of the membrane are hydrophilic while the interior is hydrophobic, so the increased lipophilicity of (2*E*)-alkenals should affect their movement further into the membrane lipid bilayer portions. It should be logical to assume that most of the lipophilic (2*E*)-alkenal molecules being dissolved in the medium are partially incorporated into the lipid bilayers (Franks and Lieb, 1986) in which they may react with biologically important substances. The amount of (2*E*)-alkenals entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. Hence, the length of the alkyl chain is associated with eliciting activity to a large extent (Kubo and Kubo, 1995; Kubo et al., 1995a).

3. Polygodial

A bicyclic sesquiterpene dialdehyde, polygodial (**1**), was first isolated as a pungent principle from the sprout of *Polygonum hydropiper* (Polygonaceae) (Barnes and Loder, 1962; Ohsuka, 1963), known as 'tade' and used as a food spice in Japan. Its congener, warburganal (**2**), was isolated from two East African *Warburgia* trees together with polygodial in minute amounts (Kubo et al., 1976) and these two plants are also locally used as food spice (Watt and Breyer-Brandwijik, 1962). Their potent fungicidal activity, especially against yeasts such as *C. albicans* and *S. cerevisiae*, was subsequently reported, although they possessed little or no activity against bacteria (McCallion et al., 1982; Taniguchi et al., 1984). In previous reports, the antibacterial activity of polygodial was tested against only a few selected bacteria and the highest concentration tested was 100 μg/ml, because of polygodial's potent antifungal activity (Taniguchi et al., 1984). The antifungal activity of polydial studied using *S. cerevisiae* as a model was found to involve its multifunction. Polygodial first acts as a surface-active agent (surfactant) and is then involved in biochemical processes. The primary antifungal action of polygodial comes from its ability to act as a surfactant, which thus disrupts the lipid-protein interface of integral proteins nonspecifically, denaturing their functioning conformation. On the basis of the surfactant concept found mainly against *S. cerevisiae*, polygodial can be expected to possess a broad spectrum due to its nonspecific mechanism. Hence, polygodial was reexamined against the selected bacteria including *S. choleraesuis* and found to show moderate antibacterial activity (Kubo and Fujita, 2001). Subsequently, polygodial was found to exhibit moderate antibacterial activity against four common Gram-negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Proteus vulgaris* (Kubo et al., 2005). Since in the previous experiments, polygodial did not exhibit any antibacterial activity against these Gram-negative bacteria up to 100 μg/ml, the result obtained was unexpected.

Polygodial exhibited the activity against *S. choleraesuis* with both MIC and MBC of 50 μg/ml (170 μM), suggesting that no residual bacteriostatic activity is involved. The bactericidal activity was confirmed by the time kill curve experiment as shown in Figure 5. Cultures of *S. choleraesuis*, with a cell density of 4.4×10^4 CFU/ml, were exposed to three different concentrations of polygodial. The number of viable cells was determined following different periods of incubation with polygodial. It shows that MIC significantly reduced the growth rate, but that the final cells count was not different from the control. It should be noted that lethality occurred quickly, within the first 1 h after adding polygodial. This rapid lethality very likely indicates that antibacterial activity of polygodial against *S.*

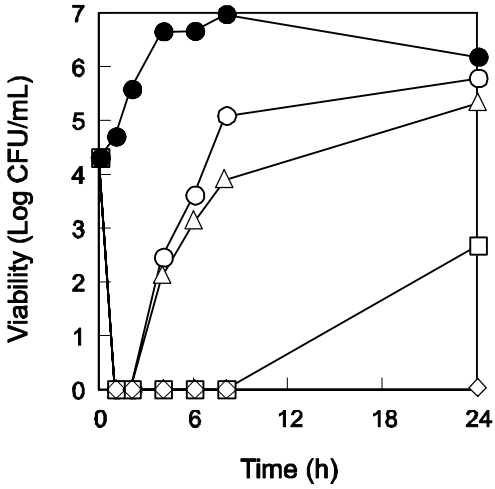

Fig. 5. Bactericidal effect of polygodial against *S. choleraesuis* subsp. *choleraesuis*. Exponentially growing cells of *S. choleraesuis* were inoculated into NYG broth with 0 (●); 12.5 (○), 25 (△), 50 (□) or 100 (◇) μg/ml of polygodial. Incubation was done at 37°C without shaking. Viability was estimated by the number of colonies formed on NYG plate after incubation at 37°C for 24 h.

choleraesuis is associated with the disruption of the membrane, similar to its effect found against *S. cerevisiae* (Kubo et al., 2001).

In previous reports, the antifungal activity of polygodial against *S. cerevisiae* was described to be significantly enhanced in combination with anethole (**3**) (Kubo and Himejima, 1992). Hence, polygodial was combined with anethole to see if the same combination effect can also be observed against *S. choleraesuis*. Anethole itself exhibits antibacterial activity against this food-borne bacterium with both MIC and MBC of 200 μg/ml (1350 μM). Similar to polygodial and (2*E*)-hexenal, no differences in MIC and MBC were noted, suggesting that no residual bacteriostatic activity was involved. The bactericidal effect of anethole was confirmed by the time kill curve method as shown in Figure 6. The lethality occurred slower than that of polygodial, 4 h after the addition of anethole. It shows that ½MIC reduced the growth rate, but that the final cell count was not significantly different from the control. *S. choleraesuis* is one of the few Gram-negative susceptible bacteria to anethole, which thus resembles polygodial.

The combination of polygodial and anethole synergistically retarded the growth rate of *S. choleraesuis* to a large extent, but this combination showed only marginal synergism on their bactericidal action. Thus, *S. choleraesuis* cells appeared to adapt to this combination stress, eventually recovering and growing normally. These results may indicate possibly

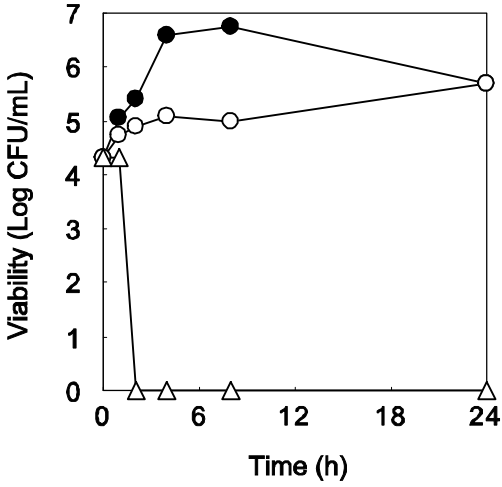

Fig. 6. Bactericidal effect of anethole against *Salmonella choleraesuis* subsp. *choleraesuis*. Exponentially growing cells of *S. choleraesuis* were inoculated into NYG broth with 0 (●); 100 (○), 200 (△) μg/ml of anethole. Incubation was done at 37°C without shaking. Viability was estimated by the number of colonies formed on NYG plate after incubation at 37°C for 24 h.

different antimicrobial mechanism of the combination between yeasts and bacteria, or more specifically between *S. cerevisiae* and *S. choleraesuis*. Anethole was also combined with (2*E*)-hexenal to see if the combination has any enhancing activity. This combination also exhibited strong synergism on their bacteriostatic action, but only marginal synergism on their bactericidal action. The reason for the residual bacteriostatic activity against *S. choleraesuis* remains unknown.

4. Alkanols

Since α,β-unsaturated aldehydes are chemically highly reactive substances, some practical application may limit the scope of the use of (2*E*)-alkenals or polygodial as anti-*Salmonella* agents. On the other hand, alkanols are considered to be chemically stable, and hence, a series of aliphatic alkanols from C_5 to C_{14} were tested for their antibacterial activity against *S. choleraesuis*. The results are listed in Table 3 and are basically similar to those observed for the corresponding (2*E*)-alkenals. The range of the antibacterial activity of the alkanols tested against this food-borne bacterium is between 6.25 and 800 μg/ml, and MICs and MBCs are the same. Dodecanol (C_{12}) was found to be the most effective against *S. choleraesuis* with an MBC of 6.25 μg/ml (34 μM), followed by undecanol

(C_{11}) with an MBC of 12.5 µg/ml (67 µM). Hexanol (C_6) did not exhibit any activity against *S. choleraesuis* up to 1600 µg/ml. Notably, no alkanol exhibits any antibacterial activity against the other Gram-negative bacteria tested, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Proteus vulgaris* and *Helicobacter pylori*. They are all resistant to alkanols and this may be caused by their different permeability of the outer membrane layer since this plays a major role in the general resistance of Gram-negative bacteria especially to lipophilic antibiotics.

Table 3. Antibacterial activity (µg/ml) of alkanols against *Salmonella choleraesuis* subsp. *choleraesuis* ATCC 35640

Alkanols Tested	*MIC (MBC)*
C_5	>1600 (*>1600*)
C_6	>1600 (*>1600*)
C_7	800 (*800*)
C_8	400 (*400*)
C_9	200 (*200*)
C_{10}	50 (*50*)
C_{11}	12.5 (*12.5*)
C_{12}	6.25 (*6.25*)
C_{13}	6.25-50* (*6.25-100*)*
C_{14}	>100 (*>100*)

Numbers in *italic* type in parenthesis are *MBC.*
(*) The values are variable.

The bactericidal effect of dodecanol was also confirmed by the time kill curve method as shown in Figure 7. Cultures of *S. choleraesuis*, with a cell density of 5×10^5 CFU/ml, were exposed to two different concentrations of dodecanol. The number of viable cells was determined following different periods of incubation with dodecanol. The result verifies that MIC and MBC are the same. It shows that ½MIC slowed growth, but that the final cell count was not significantly different from the control. In the time kill curve experiment, lethality occurred notably quickly, within the first 1 h after adding dodecanol. This rapid lethality very likely indicates that the antibacterial activity of dodecanol against *S. choleraesuis* is associated with the disruption of the membrane, similar to its effect described against *S. cerevisiae* (Kubo et al., 2003b).

Subsequently, the effect of dodecanol on the growth of *S. choleraesuis* treated with chloramphenicol was examined. Chloramphenicol restricted cell division thereby inhibiting bacterial protein synthesis. In fact, cell

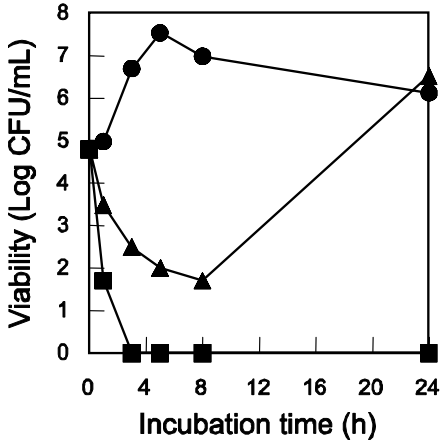

Fig. 7. Effect of dodecanol on the growth of *S. choleraesuis* subsp. *choleraesuis*. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. The arrow indicates the time when drug was added. Dodecanol, 0 (●), 3.13 (▲), and 6.25 (■) μg/ml.

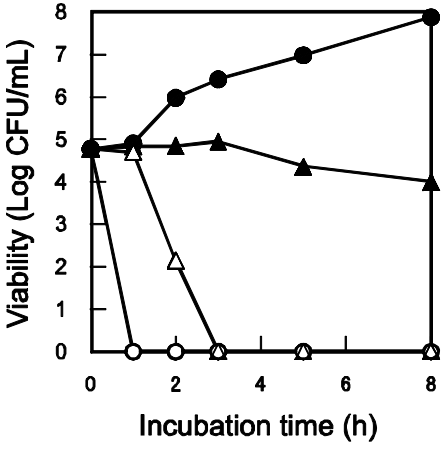

Fig. 8. Effect of dodecanol on the growth of *S. choleraesuis* subsp. *choleraesuis* in the presence of chloramphenicol. Exponentially growing cells were inoculated into NYG broth and then cultured at 37°C. Chloramphenicol at 0 (●, ○), and 6.25 (▲, △) μg/ml was added to each culture at 0 h. Dodecanol at 12.5 μg/ml was added at 0 (○) and 1 (△) h.

viability of *S. choleraesuis* in the presence of 6.25 μg/ml of chloramphenicol was kept at the same level during incubation as shown in Figure 8. Dodecanol reduced the viability rapidly regardless of the treatment of chloramphenicol. Hence, the bactericidal effect of dodecanol is not thought to be the necessary function accompanying reproduction of *S. choleraesuis* cells, which involves macromolecule biosyntheses such as DNA, RNA and protein, and cell wall synthesis.

The antibacterial activity of medium chain (C_9-C_{12}) alkanols against *S. choleraesuis* was mediated primarily due to their nonionic surface-active properties disrupting lipid-protein interface, and the maximum activity can be obtained when balance between the hydrophilic and hydrophobic portions becomes the most appropriate. This surfactant concept can be supported by the observation that the potency of the activity against *S. choleraesuis* was distinctly increased with each additional CH_2 group, up to dodecanol. As abovementioned for medium chain alkanols, first approach the binding site with the electron negativity of the hydroxyl oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayers. For example, the hydroxyl group of cholesterol resides near the membrane-water interface in the lipid bilayers and is likely to be bonded with the carbonyl group of phospholipids. Alkanols may function by disrupting and disorganizing the hydrogen bonds. Cholesterol is a major component of the plasma membrane and owes its membrane-closing properties to its rigid longitudinal orientation in the membrane. Cholesterol has profound influences on membrane structure and function, therefore, if the hydrogen bond is broken, cell function will be impaired. Hence, it is possible to suggest that alkanols also act at the lipid-protein interface of integral proteins nonspecifically, such as ion channels and/or transport proteins, denaturing their functioning conformation, in a similar manner described against *S. cerevisiae*. The common nature among these alkanols should be considered in that the electron negativity on the hydroxyl oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be increased maximally by hydrophobic compounds of particular hydrophilic hydroxyl group (Fujita and Kubo, 2005a). Thus, the medium-chain alkanols disrupt the hydrogen bonding in the lipid-protein interface in *S. choleraesuis*. The data obtained are consistent with an effect on the bulk membrane rather than a direct interaction of the specific target protein and alkanols' non-specificity of antimicrobial activity supports this assumption. The possibility of the anti-*Salmonella* activity of the medium-chain alkanols is due to their nonionic surfactant property, but this may not be the case for short-chain alkanols. The short chain alkanols enter the cell by passive diffusion across the plasma membrane

and/or through porin channels. On the other hand, the more lipophilic long chain alkanol molecules are incorporated in part into the lipid bilayers. The amount of alkanols entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. Nonetheless, alkanols are chemically stable compounds and may not react with any biologically important substances in the cytosol or lipid bilayer. Hence, the primary antibacterial action of medium-chain alkanols comes from their ability to function as nonionic surfactants (physical disruption of the membrane). It can be concluded that the medium-chain alkanols target the extracytoplasmic region as surfactants. This is highly desirable since they do not need to enter the cell, thus, avoiding most cellular pump-based resistance mechanisms. In addition, alkanols are considered to be chemically stable, colorless, inexpensive, biodegradable and essentially non-toxic to humans. Therefore, the medium-chain alkanols can be used for food as anti-*Salmonella* agents.

5. Discussion

The study shows that polygodial as well as medium-chain (2*E*)-alkenals and alkanols are bactericidal against *S. choleraesuis* and kills the cells quickly. Since polygodial possesses potent antifungal activity, its mode of action has been extensively studied using *S. cerevisiae* as a model. In brief, the fungicidal activity of polygodial is exerted by its multiple functions but primarily comes from its ability to act as a nonionic surfactant, thereby disrupting lipid-protein interface (Kubo et al., 2001a; Fujita and Kubo, 2005b). This surfactant concept can also be applicable in part against *S. choleraesuis* because the lethality against this food-borne bacterium occurred remarkably quickly in the time kill experiment, within the first 1 h after adding polygodial. This rapid lethality observed supports its ability to function as a nonionic surfactant. If this is the case, polygodial very likely targets the extracytoplasmic region as a nonionic surfactant and thus does not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. A further study to investigate this idea was not performed since polygodial falls short of the broad spectrum of activity as far as Gram-negative bacteria are concerned. *S. choleraesuis* was the rare susceptible Gram-negative bacterium to polygodial, indicating that *S. choleraesuis* differs from other Gram-negative bacteria in some aspects. This difference may be caused by their different permeability of the outer membrane layer since this layer in Gram-negative bacteria plays a major role in the general resistance against drugs. In general, antibacterial activity against Gram-negative bacteria decreases by increasing the lipophilicity of molecules. However, the antibacterial agents against *S. choleraesuis* characterized so far are not the case, but they are rather similar to those

against Gram-positive bacteria and fungi.

In contrast to polygodial, (2*E*)-hexenal is noted to possess a broad antimicrobial spectrum (Muroi et al., 1993; Kubo et al., 1996). Although the precise mode of antimicrobial action of this alkenal is not yet clear, it should be nonspecific mechanism due to its broad spectrum. (2*E*)-hexenal unlikely acts as a surfactant but likely permeates by passive diffusion across the plasma membrane. Once inside cells, its α,β-unsaturated aldehyde moiety readily reacts with biologically important nucleophilic groups. For example, this aldehyde moiety is known to react with sulfhydryl groups mainly by 1,4-additions under physiological conditions (Schauenstein et al., 1977). Sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell. Limited to this reactivity, (2*E*)-hexenal and polygodial resemble. However, the precise target molecule remains unclear.

In our continuing search for antimicrobial agents from plants, a number of active principles have been characterized. However, only a few of them showed activity against Gram-negative bacteria, especially the *Pseudomonas* species. Among the compounds we characterized as antibacterial agents, (2*E*)-hexenal is one of the two phytochemicals characterized as antibacterial agents against *Pseudomonas aeruginosa*. We first characterized (2*E*)-hexenal as the principal antimicrobial agent from the cashew apple and subsequently olive oil. This common α,β-unsaturated aldehyde is known as 'leaf aldehyde' (Hatanaka, 1993) and is widely distributed. It may be a key defense chemical (postinhibitin) against microbial attacks.

The antibacterial activity of (2*E*)-hexenal against *E. coli*, *P. aeruginosa*, *E. aerogenes*, and *P. vulgaris* (Kubo et al., 1996) as well as *H. pylori* (Kubo et al., 1999) was previously reported. Due to this broad antimicrobial activity and availability in many edible plants (Schauenstein et al., 1997), this aliphatic α,β-unsaturated aldehyde known as 'leaf aldehyde' (Hatanaka, 1993), was studied in more detail. In our continuing search for antimicrobial agents from edible plants, (2*E*)-hexenal was also previously characterized from the volatile fraction of the cashew apple (Muroi et al., 1993) and olive oil (Kubo et al., 1995a). In contrast to (2*E*)-hexenal, hexanol did not show any activity up to 1600 μg/ml, but hexanal and hexanoic acid still exhibited some activity, though to a lesser extent than (2*E*)-hexenal. Thus the conjugated double bond is not essential to elicit the activity although it increases it.

Currently, no appropriate anti-*Salmonella* agent for pepper is available. Hence, the phytochemicals characterized as anti-*Salmonella* agents can be applicable to disinfect and prevent the contamination. In the case to disinfect the *Salmonella* contaminated pepper, volatile α,β-unsaturated aldehydes, specifically (2*E*)-hexenal seems to fit nicely for the purpose. This

is currently under examination and the result will be reported elsewhere. On the other hand, anti-*Salmonella* phytochemicals can be mixed into artificial fodder to eliminate *Salmonella* in their sources.

Furthermore, it may be worthwhile to consider the anti-*Salmonella* activity of rather common phytochemicals from an ecological point of view. For example, it should be remembered that chickens peck green leaves. The green leaves contain a variety of antibacterial agents against *Salmonella* bacteria, especially those known as green leaf aldehydes and alcohols (Hatanaka, 1993). This indicates that *Salmonella* are very likely controlled in nature when chickens were continuously fed green leaf based food. In the Amazon basin, *Salmonella* contamination of post-harvest pepper has been increasingly noted with increasing large-scale poultry farms. This may be caused by shifting their foods from plant-based natural foods to artificial fodders. (2*E*)-Alkenals and alkanols may have potential as crop preservatives to inhibit or prevent the growth of *Salmonella* bacteria. For example, a minute amount of the medium-chain (2*E*)-alkenals such as (2*E*)-dodecenal and (2*E*)-undecenal can be added to the artificial fodder. On the other hand, high concentrations are needed to cause the loss of viability, but (2*E*)-hexenal may be considered as a genuine anti-*Salmonella* agent because of its high volatility (Wilson and Winiewski, 1989; Corbo et al., 2000) and wide distribution in many edible plants such as, apples, pears, grapes, strawberries, kiwi, tomatoes, olives, etc (Schauenstein et al., 1977).

Safety is a primary consideration for anti-*Salmonella* agents, especially those in food products. The anti-*Salmonella* agents isolated from plants being used as food spices and/or characterized, as flavor substances in many edible plants should be superior as compared to non-natural ones. The knowledge obtained may provide insights into bactericidal action of aldehydes and alkanols on a molecular basis, and a more rational and scientific approach to use or design efficient and safe anti-*Salmonella* agents. On the basis of the data obtained, the hydrophilic aldehyde group can be replaced by any hydrophilic groups as long as the 'head and tail' structure is balanced. Hence, various additional biological activities can be introduced mainly by selecting appropriate head portions. For example, a series of each alkyl gallates (Fujita and Kubo, 2002; Kubo et al., 2002a) and alkyl protocatechuates (Nihei et al., 2003; Nihei et al., 2004) were synthesized as antioxidative anti-*Salmonella* agents.

6. Antibacterial bioassay

The test strain, *Salmonella choleraesuis* subsp. *choleraesuis* ATCC 35640 was purchased from the American Type Culture Collection (Manassas, VA). NYG broth (0.8% nutrient broth, 0.5% yeast extract, and 0.1% glucose) was used for the antibacterial assay. The nutrient broth was purchased from

BBL Microbiology System (Cockeysville, MD). The yeast extract was obtained from Difco Lab (Detroit, MI).

The cells of *S. choleraesuis* subsp. *choleraesuis* ATCC 35640 were precultured in 3 ml of NYG broth without shaking at 37°C for 16 h. The preculture was used for the following antibacterial assay and time kill study. The test compounds were first dissolved in DMF and the highest concentration tested was 1600 µg/ml. It should be noted however that higher concentrations reported might not be accurate because of their solubility limitation in the water-based medium. The final concentration of DMF in each medium was 1%, which did not affect the growth of the test strain. Broth macrodilution methods were used as previously described (Kubo et al., 1995a) with slight modifications. Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF, and 30 µl of each dilution was added to 3 mL of NYG broth. These were inoculated with 30 µl of preculture of *S. choleraesuis*. After the cultures were incubated at 37°C for 24 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound that demonstrated no visible growth. The MBC was determined as follows. After the determination of the MIC, 100-fold dilutions with drug-free NYG broth from each tube showing no turbidity were incubated at 37°C for 48 h. The MBC was the lowest concentration of the test compound that showed no visible growth in the drug-free cultivation.

The bactericidal activity of the selected compounds was confirmed by the time kill curve experiment. The cultivation with each compound was done the same as the above MIC assay. Samples were withdrawn at selected time points, and serial dilutions were performed in sterile saline before the samples were plated onto NYG agar plates. After the plates were incubated at 37°C for 16 h, colony forming units (CFU) were estimated.

It should be noted that the MIC and MBC values against *S. choleraesuis* were noted to be variable in some degree. The maximum extent and rate of antimicrobial activity is known to vary with the experimental conditions such as the seed culture mediums, the physiological age of the culture, and the type of culture medium. The variation observed may be caused in part by volatilization of the test compounds from the test medium during the incubation. This postulate can be supported by the observation that the nonvolatile compounds tested, such as polygodial, were relatively constant compared to that of volatile (2*E*)-hexenal. Nonetheless, the activity against *S. choleraesuis* seems to be more affected in general by experimental conditions compared to other microorganisms.

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4 Natural Chemotherapeutic Agents in the Control of Malaria

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Introduction

Huge efforts are continuously being made in finding effective remedies for the control of malaria. Globally, malaria remains one of the most infectious diseases, which is also life threatening, infects about half a billion people resulting in the death of between 1.5 and 2.7 persons, annually with a high mortality rate among children (WHO, 1997, 2000a; Snow et al., 2001). The observed increase in morbidity and mortality is attributed to the loss of effectiveness of readily available and effective chemotherapeutic agents to the malaria parasite (Ollairo and Bloland, 2001). Malaria is caused by the apicomplex parasite, *Plasmodium*. It has four main species affecting humans namely: *Plasmodium falciparum*, *P. vivax*, *P. malaraie* and *P. ovale*. By far, *P. falciparum* is the most virulent and widespread etiological agent for human malaria.

The disease occurs in well over 100 countries and territories. More than 40% of the earth's population is at risk including large areas of Africa, Central and South America, South East Asia and the Middle East. Over 90% of the cases of malaria occur in Sub-Saharan Africa and this has contributed in no small measure to increased mortality and economic hardships in the developing world. In USA, about 1200 cases of malaria are diagnosed yearly (Roll back malaria, 1998; Marsh, 1998; Breman, 2001; Ohaeri, 2004).

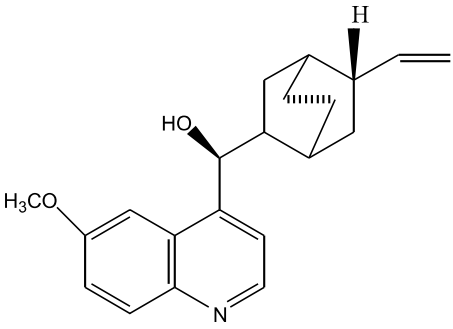
The importance of plants as a source of new antimalarial as well as vector control agents is the highlight of this chapter. The urgent need to

continue to seek new entities for malaria control even as malaria vaccine development is being awaited is also discussed.

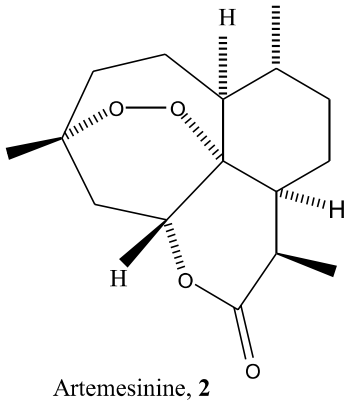
Various proposals are identified in the control of malaria and most governments in Africa advocate the need for an integrated approach. Prompt diagnosis, the use of chemotherapeutic agents, vector control, improved management and search for an effective vaccine are some of these approaches (Okenu, 1999).

Today, malaria is still one of the diseases for which not many appropriate drugs are available. Various reasons can be ascribed to this deficiency. This includes insufficient efficacy and increasing loss of effectiveness to readily available drugs, due to resistance by the parasite, *P. falciparum*. Consequently, therapeutic agents against malaria are continuously being sought. Effective drugs are urgently needed and will still be needed in the near future (Canianto and Puricelli, 2003). In the course of the past few years, scientific progress in chemotherapy of malaria has focused on existing drugs, rather than development of new ones. The need to develop new antimalarial drugs is of high priority because of the development of resistance by the parasites to cheap first line drugs in both uncomplicated and severe malaria. In addition, recrudescence observed in the use of some newer antimalarial drugs has led to the use of drug combination with the hope of achieving delay or total circumvent to resistance (Sowunmi et al., 1997; WHO, 2000b). Antimalarial drug discovery is dependent on identification of new targets. With the advent of improved research on molecular biology, the sequencing of *P. falciparum* genome though highly laborious has been successfully completed. This will definitely facilitate genomic approaches to drug discovery (Olliaro and Bloland, 2001).

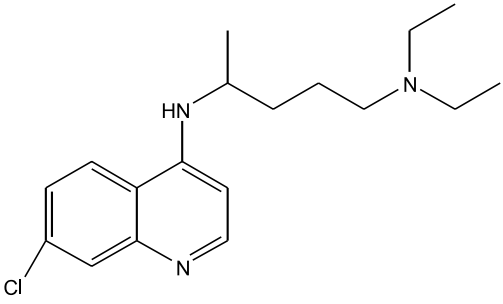
Ultimately, plants have been a rich source of new chemotherapeutic agents and some classical antimalarial drugs in use today: quinine **1** and artemisinin **2** were either obtained directly from plant sources or developed from plant compounds (Klayman, 1985; Dhingra et al., 2000). With increasing resistance to antimalarial drugs like chloroquine **3** and mefloquine **4**, there is certainly the dire need to find newer and effective drugs (Ridley, 2002). In contributing to the discovery of natural chemotherapeutic agents that could be developed for the control of malaria, compounds found in literature (Olliaro and Yuthavong, 1999; Schwikkard and van Heerden, 2002; Canianto and Puricelli, 2003; Kumar et al., 2003) in addition to the research findings of our group, the malaria research group, University of Ibadan, Nigeria, until 2004 are presented. Presently, no vaccines have been developed and there are no prospects for vaccines being available soon enough! Until such time, chemotherapeutic agents still remain the major form of control of malaria, a most devastating disease.



Quinine, **1**



Artemesinine, **2**



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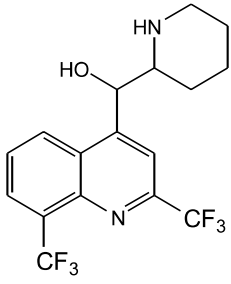


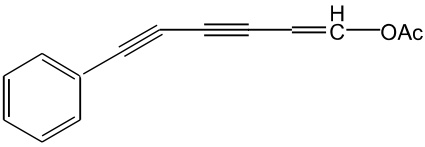
Mefloquine, **4**

Natural Products in Malaria Chemotherapy

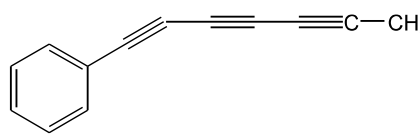
Acetylenes

Phenyl acetylene **5** and phenyl heptatriyne **6**, isolated from the roots of *Bidens pilosa* (Asteraceae) have been reported to be active against *P. falciparum*. Evaluation of nine species of *Bidens*, for antimalarial properties indicated that the acetylenic compounds were probably associated with flavonoids in the roots of the plants (Brandao et al., 1997, 1998). Minquartynoic acid **7**, isolated from the South American plant *Minquartia guinensis* (Olacaceae) is an aliphatic acid with four ethynic bonds. It was found to be active in the *in vitro* antiplasmodial bioassay-led fractionation of *M. guinensis*. It however failed to elicit any selectivity to the malaria parasite, as it was found to be toxic in the cytotoxicity studies (Rasmussen et al., 2000).

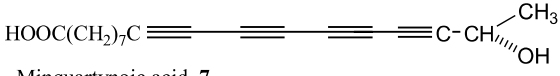
Acetylenes



Phenyl acetylene, **5**



Phenyl heptatriyne, **6**

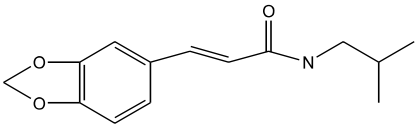


Minquartynoic acid, **7**

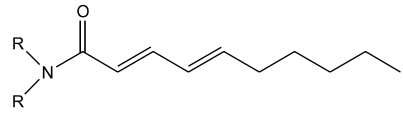
Amides

The root bark of a Tanzanian medicinal plant, *Zanthoxylum gilletti* (Zanthoxylaceae), in the work reported by Weenen et al. (1990) furnished the antiplasmodial compounds, fagaramide (IC_{50} = 12.34 µg/ml) **8** and N-isobutyldeca-2,4-dieneamide **9** (IC_{50} = 5.37 µg/ml). Deca-2, 4-dienamide (E, Z), **10** (IC_{50} of 192.8 µM), isolated from *Phyllanthus fraternus* (Euphorbiaceae) was also found to display moderate *in vitro* antiplasmodial activity (Sittie et al., 1998). The root bark of the Hazalea plant, *Fagara rhetza* (Rutaceae) yielded the amide, hazaleamide **11**, which displayed good activity against chloroquine resistant *P. falciparum* as reported by Shibuya et al. (1992).

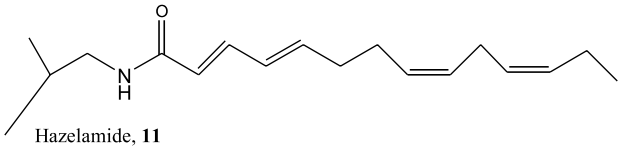
Amides



Fagaramide, **8**



N- Di isobutyldeca-2,4-dieneamde, **9** (R = isobutyl)
(E,Z)-Deca-2,4- dieneamide, **10** (R = H)



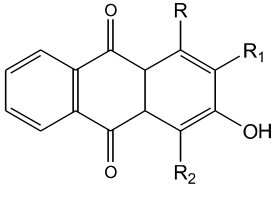
Hazelamide, **11**

Anthraquinones

From *Morinda lucida* (Rubiaceae), a popularly used remedy for the management of malaria in West Africa, three compounds digitolutine **12**, rubiadin-1-methyl ether **13**, and damnacanthal, **14** were isolated from the stem bark and roots of the plant. The antimalarial properties of the compounds were investigated on the growth of *P. falciparum* using the schizont inhibition assay. They displayed a dose-dependent activity attaining 100% inhibition with 30-40 µg of compounds. Damnacanthal, **14** showed the highest activity and this was attributed to the aldehyde group at position C-2 (Koumaglo et al., 1992; Sittie et al., 1999).

From the Nigerian phytomedicine, emodin **15**, was identified as the antimalarial principle in extracts of both *Cassia nigricans* and *C. siamea* stem bark (Fabaceae), respectively. In the antiplasmodial assay using the multi-resistant strain of *P. falciparum* K1, it had IC_{50} of 5 µg/ml in the parasite lactate dehydrogenase assay (Obodozie et al., 2004; Ajaiyeoba et al., 2007).

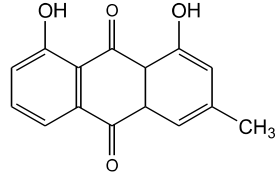
Anthraquinones



Digitolutine, **12:** R= H; $R_1 = CH_3$; $R_2 = OCH_3$;

Rubiadin-1-methyl ether, **13:** R= OCH_3;
$R_1 = CH_3$; R_2 = H

Damnacanthal **14:** R= OCH_3; R_1 = CHO; R_2 = H



Emodin, **15**

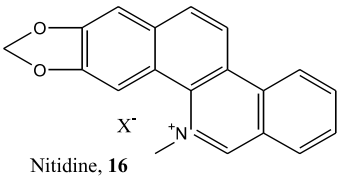
Alkaloids

Several alkaloids have displayed *in vitro* antiplasmodial properties as well as in animal models. Ever since the identification and development of quinine **1**, a quinoline alkaloid, there has been tremendous effort to source plants for alkaloids with antimalarial properties. The reason in so doing is to enhance structural novelty (Hadi and Bremner, 2001). The quinine molecule inspired the synthesis of chloroquine **3**, which became the drug of choice for chemotherapy of malaria until the advent of notable resistance. Several alkaloids with diverse structures have displayed antiplasmodial properties. This includes, aporphine, bisbenzylisoquinoline, indole and

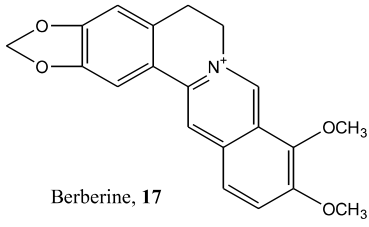
bisindole, isoquinoline, indoloquinoline, naphthylisoquinoline and quinoline alkaloids as outlined below.

Aporphine alkaloids—Nitidine **16** isolated from *Toddalia asiatica* (Rutaceae), though cytotoxic, showed good activities against sensitive and resistant strains of *P. falciparum* (Gakunju et al., 1995). Twenty-one alkaloids were assessed for activities against *P. falciparum* (multi resistant strain, K1) *in vitro*. Only two of the total alkaloids investigated, berberine **17** and dehydrodiscretine **18**; displayed antiplasmodial properties with IC_{50} < 1 μM (Omulokoli et al., 1997; Wright et al., 2000). Berberine has also been known to be effective against trypanosomes (Freiburghaus et al., 1996). Roemrefidine **19**, isolated from *Sparattanthelium amazonum* (Hernandiaceae) was found to be active against resistant and sensitive strains of *P. falciparum in vitro*. It also inhibited *P. berghei* in mice in addition to being non-toxic in three cell lines (Munoz et al., 1999).

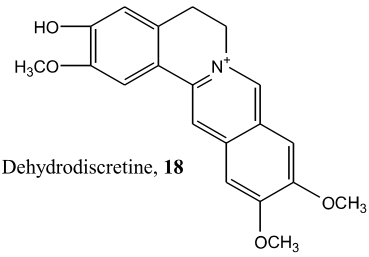
Aporphine Alkaloids and Derivatives



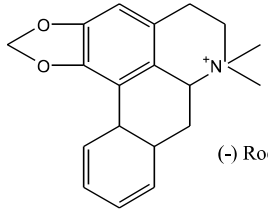
Nitidine, **16**



Berberine, **17**



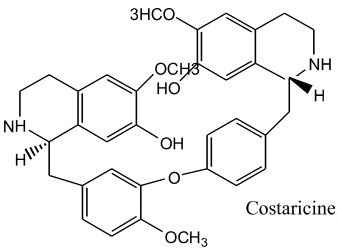
Dehydrodiscretine, **18**



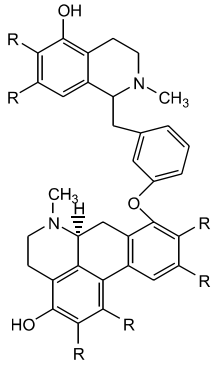
(-) Roemefidine, **19**

Bisbenzylisoquinoline alkaloids—Studies on the Costa Rican plant *Nectandra salicifolia* (Lauraceae) led to the isolation of sixteen alkaloids with only one of them costaricine **20,** displaying intrinsic antimalarial activity against *P. falciparum* (Bohlke et al., 1996). Bisbenzyl isoquinoline alkaloids have been reported from the Chinese medicinal herbs, *Thalictrum fabeni* (Rananculaceae) and *Cyclea barbata* (Menispermaceae). Three of such alkaloids were evaluated for antimalarial properties. Though thalifacine **21,** most found to be most active, it was also found cytotoxic against human

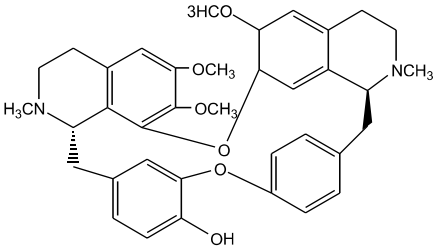
Bisbenzylisoquinoline Alkaloids



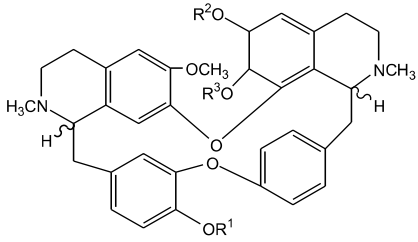
Costaricine, **20**



Thalifasine, **21** R=OCH_3



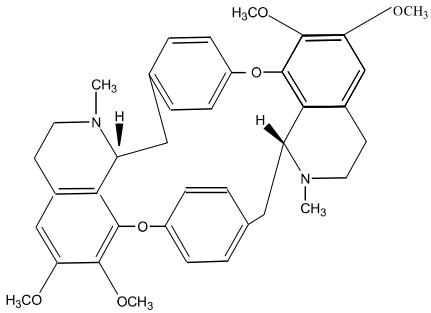
Penduline, **22**



Candicusine, **23** - R^1 =H, R^2=CH_3, R^3=H 1*R*, 1′*R*

Cepharanthine, **24** - R^1=CH_3, R^2+ R^3= 1R,1′*S*

Cycleapeltine, **25** - R^1=CH, R^2=CH, R^3=H, 1*S*,1′*S*



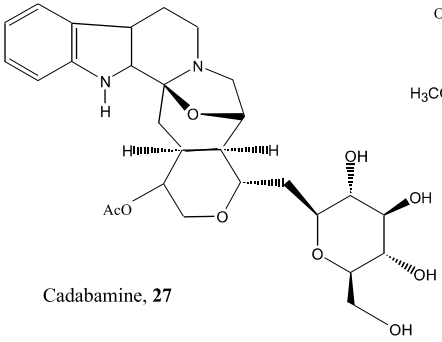
Cycleamine, **26**

cell lines (Lin et al., 1993). Valentin et al. (1997) also isolated penduline **22**, from *Isopynum thalictriodes* (Ranunculaceae) and it was found to be active against chloroquine-resistant strains of *P. falciparum*. Several bisbenzyl isoquinoline alkaloids from a variety of plant families (check references Steele et al., 1999; Likhitwitayawuid et al., 1993a, 1993b) were screened for antiplasmodial properties. Of a total of 53 alkaloids that were screened using the chloroquine sensitive and resistant strains (D6 and W2), respectively, candicusine **23**, cepharanthine **24**, were most active against the D6 strain, while the most active against the W2 strain was cycleapentine **25**. Cycleamine**, 26** was the most selective in the study, apart from being the most potent compound, it also had the least toxicity cytoxocity assay, it was active in the *in vivo* animal studies for malaria. Cycleanine is a good candidate for drug development. There have been reports of bisbenylisoquinolines acting as modulators to chloroquine resistance in *P. falciparum* investigations *in vitro* (Tian and Pan, 1997; Hakuri et al., 2000). There is need for these compounds to be evaluated in laboratory animals. In which case these could be worth considering for drug development.

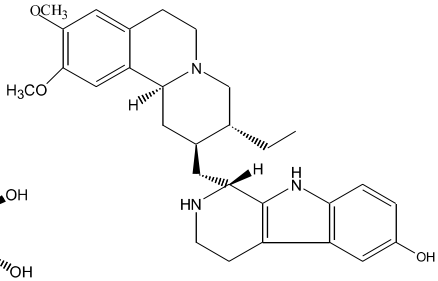
Indole alkaloids and derivatives—Cadabamine, **27**, isolated from an Asian plant, *Anthocephalus chinensis* (Rubiaceae) was shown by Kitagawa et al. (1996a) to have activity against a chloroquine resistant strain of *P. falciparum* with an IC_{50} value of 6.8 μM. From the Bolivian ethnomedicinal plant, *Pogonopus tubulosus* (Rubiaceae) three antiplasmodial indole alkaloids: cephaline, psychotrine and tubulosine **28** were identified. Tubulosine **28**, was the most active in the assays and showed an IC_{50} values of 0.006 μg/ml and 0.011 μg/ml on chloroquine-sensitive and resistant strains, respectively. It was also found to be non-toxic using the KB cells cytotoxicity assay. It elicited activity in the *in vivo* animal antimalarial analysis using *P. vinckei petteri* and *P. berghei* and was active at doses that were not lethal. Tubulosine **28** seems to have promise as a suitable candidate for drug development (Sauvain et al., 1996).

Strychnos (Strychnaceae) species have furnished the indole alkaloids, icajine, isoretuline and strychnobrasiline **29,** which though did not display antiplasmodial properties *in vitro* were able to reverse *P. falciparum* activity at a concentration range of 2.5-25 μg/ml (Frederich et al., 2000). Vocamine **30**, a bisindole alkaloid isolated initially from *Voacanga* species and more recently from *Peschiera fuchsiaefolia* (Apocynaceae), exhibited *in vitro* antiplasmodial activities against chloroquine sensitive D_6 (IC_{50} = 0.238 μg g/ml) and chloroquine resistant W_2 (IC_{50} = 0.290 μg/ml) strains respectively. These activities were more pronounced than those of the reference drugs included in the assay (Federici et al., 2000). It also displayed interesting *in vivo* activities though it was less than that of the reference compounds and there was low toxicity against mouse and human cell

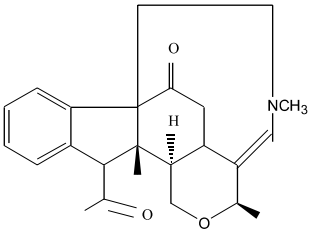
Indole Alkaloids



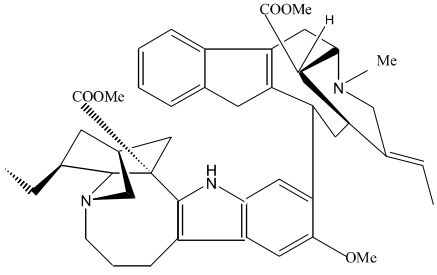
Cadabamine, **27**



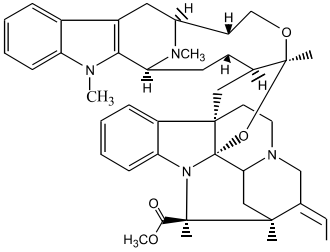
Tubulosine, **28**



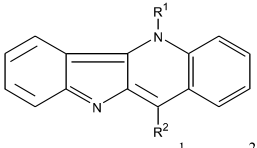
Strychnobrasiline, **29**



Vocamine, **30**



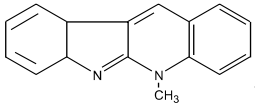
Villastonine, **31**



Cryptolepine, **32** - R^1=CH, R^2=H

11Hydroxycryptolepine, **33** - R^1=CH_3 R^2=OH

Quindoline, **34** - R^1=H, R^2=H



Neocryptine, **35**

lines. It has been suggested that the possible mode of action of **30** is on the parasite DNA and or protein synthesis (Ramanitrahasimbola et al., 2001). Voacamine **30** is thus a candidate worthy of drug development because of the good selectivity index. From the *Alstonia* plant species (Apocynaceae), commonly found in Africa, Asia, Central America and prominently used for management of malaria in these ethnomedicines, several indole alkaloids have been isolated (Wright et al., 1993). The most active of the several alkaloids tested for antimalarial properties,

Villalstonine **31**, was identified from *A. angustifolia, A. glaucescens* and *A. macrophylla* (Keawpradub et al., 1999). Cryptolepine **32,** an indoloquinoline alkaloid, isolated from the roots of *Crytolepsis sanguinolenta* (Asclepiadaceae) a Central and West African traditional medicinal plant that has been reported to display antiplasmodial properties. The workers employed the chloroquine sensitive and resistant strains of *P. falciparum* in several studies (Kirby et al., 1995; Greiller et al., 1996; Cimanga et al., 1997; Paulo et al., 2000). Three other alkaloids were isolated from the plant species, namely: 11-hydroxycryptolepine **33**, quindoline **34** and neocryptine **35**. The details of the *in vitro* antiplasmodial and *in vivo* antimalarial properties of the four compounds **32-35** are displayed in Table 1.

Table 1. *In vitro* and *in vivo* antimalarial studies of alkaloids of *Crytolepsis sanguinolenta**

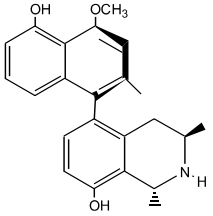
Compound	*Activity/Parasite strain*			
Name ***(Figure)***	*In vitro (P. falciparum, IC_{50} in µg/ml)*			*In vivo (P. berghei berghei % Parasitemia*
	D_6	W_2	*K1 strain***	*in mice) Anka*
Cryptolepine (**32**)	27	41	33	45.4
11-Hydroxycryptolepine (**33**)	31	52	62	43.2
Neocryptolepine (**34**)	35	65	81	–
Quindoline (**35**)	63	108	87	48.2

*Cimanga et al. (1997)

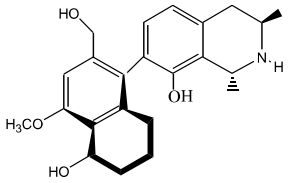
**D_6 = Chloroquine sensitive; W_2 = Chloroquine resistant; K1 = multi resistant.

Naphthylisoquinoline alkaloids—The African and Asian ethnomedicines have resulted in the bioactivity-led identification of the Dioncophyllaceae and Anchstrocladaceae plant families as sources for antimalarial agents. The *in vitro* assay, (chloroquine resistant *P. falciparum* NF 54 strain and clone A1A9) and *in vivo* animal studies (Anka strain of *P. berghei* in mice) of *Triphyophyllum peltatum* (Dioncophyllaceae) furnished dioncophylline C **36** and dioncopeltatine A, **37** as the most active compounds (Bringmann et al., 1997; Bringmann and Feineis, 2000). Further evaluations revealed that the compounds also exhibited good activity against exoerthrocytic stages of *P. berghei* in human hepatoma cells that were cultured with 10 µg/ml of the alkaloids. From the Cameroonian plant *Ancistrocladus korupensis* (Ancistrocladaceae), two novel naphthylisoquinoline alkaloids, korupensis B **38** and youndamine A **39** were evaluated (Bringmann, 2003). The alkaloids displayed exceptional activities in both the *in vitro* analysis and in animal studies. For details of their antiplasmodial activities, see Table 2.

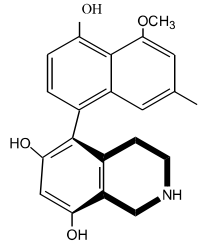
Naphthylisoquinoline Alkaloids



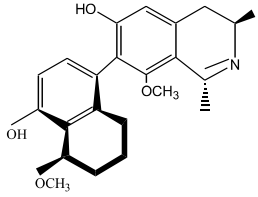
Dioncophylline C, **36**



Dioncopeltatine A, **37**



Korupsamine B, **38**



Yaoundasamine A, **39**

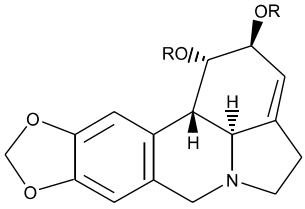
Table 2. Antimalarial properties of naphthylisoqunoline alkaloids*

Compound/Plant source *Name* ***(Figure)***	*Activity/Parasite clone*	
	*P. falciparum, IC_{50} µg/ml) NF54 clone A1A9***	*P. berghei berghei (% Parasitemia in mice) Anka*
Dioncophylline C (**36**) *Triphyophyllum peltatum*	0.014	0.00
Dioncopeltine A (**37**) *Triphyophyllum peltatum*	0.021	0.03
Korupsinamine B (**38**) *Ancistocladus korupensis*	0.041	
Youndamine A (**39**) *Ancistocladus korupensis*	2.200	

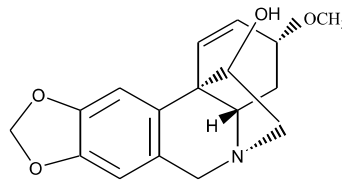
*Bringmann, 2003.
**Chloroquine sensitive clone.

Isoquinoline alkaloids—Campbell et al. (1998) identified the antimalarial principles in the Bolivian plant, *Brunsvigia littoralis* (Amaryllidaceae) to be lycorine **40**, 1,2-di-O-acetyllycorine **41,** amberlline and crinine **42** using 2 strains of *P. falciparum*. They were also evaluated for cytotoxicity using the BL_6 mouse melanoma cells. From the Madagascan antimalarial ethnomedicine, *Hernandia voyronni* (Hernandiaceae) has been reported to be used in combination with chloroquine to treat malaria

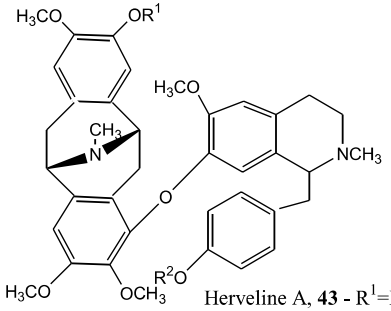
Isoquinoline Alkaloids



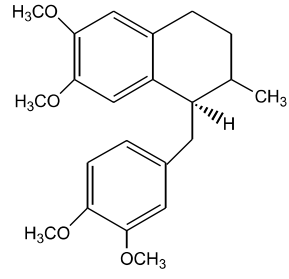
Lycorine, **40** - R=H
Acetyllycorine, **41** - R=CH_3



Crinamine, **42**



Herveline A, **43** - R^1=Me, R^2=H
Herveline D, **44** - R^1=R^2=H

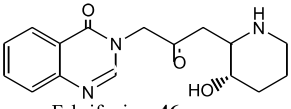


Laudanosine, **45**

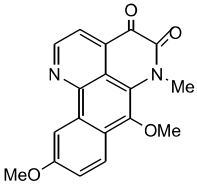
(Rasoanaivo et al., 1998). Isolation of active of the antimalarial principles led to the identification of herveline A **43** (IC_{50} 3.28 µM), herveline B, herveline C and herveline D **44** (IC_{50} 2.22 µM) and laudanosine **45** (IC_{50} 26.27 µM). These compounds displayed moderate activity against *P. falciparum*. Hervelines A, C and laudanosine enhanced chloroquine activity while compound **44** acted as antagonist (Rasoanaivo et al., 1998).

Quinazolones—Febribugine **46** and Isofebrifugine, alkaloids found in the Chinese medicinal plant, *Dichroa febrifuga* (Saxifragaceae) were the foremost alkaloids that displayed antimalarial properties. In the early studies, febrifugine **46** was a hundred times more active than quinine **1**, in the monkey model *in vivo* antimalarial assay, while compound isofebrifugine was found to be active in chicks. The toxicicological evaluation of febrifugine in mice revealed that it was toxic and so not relevant for drug development (Takaya et al., 1999). Jiang et al. (2005) evaluated 10 analogs of febrifugine **46** identifed from a febrifugine structure-based search of the Walter Reed Chemical Information System. These compounds inhibited parasite growth with IC_{50} ranging 0.141-290 ng/ml with both the chloquine-sensitive and resistant strains of *P. falciparum*. Some of these compounds displayed feasible therapeutic indices with selectivity indices >1000. One of the analogs, Halofuginone, reduced parasitemia to non-detectable levels and had curative effects in *P. berghei*

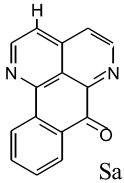
Quinazolone Alkaloids



Febrifugine, **46**



Hydranthine, **47**



Sampangine, **48**

infected-mice. Details of the IC_{50} values for four of these compounds are displayed in Table 3 below.

Two other quinazolones; hydranthine A, **47** and sampangine **48**, isolated from *Duguetia hadrantha* (Annonaceae), a South African antimalarial plant were found to demonstrate antiplasmodial properties (Muhammed et al., 2000).

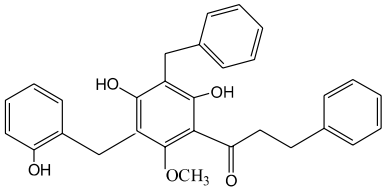
Table 3. Antimalarialand cytocoxicity properties of febrifugine ands analogs*

*Drug***	*Parasite*		*Cytotoxicity*	*Selectivity*
	P. falciparum, IC_{50} ng/ml)		*Neuronal cells*	
	W2	*D6*	*(NG108)*	
Febrifugine	0.53	0.34	63.50	119.81
Halofuginone	0.15	0.12	177.06	1222.12
WR092103	1.46	1.23	6864.29	2346.77
WR140085	15.07	12.94	15479.39	1027.00

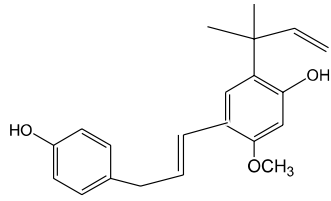
*Jiang et al. 2005; **WR092103 & WR140085 compounds from Walter Reed Chemical library.

Chalcones—Uvaretin, **49** from the Tanzanian species of *Uvaria* (Apocynaceae) was one of the foremost chalcones shown to possess antimalarial properties, though not as active as chloroquine (Pavanand et al., 1989). Phorizidine, a bitter tasting compound, isolated from *Micromelum thephrocarpum* (Rutaceae) displayed antiparasitic properties against malaria parasites. Roots of *Glycyrrhiza* sp. (licorice) popularly used in the Chinese ethnomedicine displayed antimalarial properties. Phytochemical examination of the plant, led to the identification of licochalcone A **50,** as the active principle using the chloroquine-sensitive strain (3D7) and chloroquine-resistant strain (Dd2) of *P. falciparum* in (^{3}H) hypoxanthine uptake assay. The *in vivo* evaluation against *P. yoelii* in mice, at 1000 mg/kg completely eradicated the malaria parasite without exhibiting toxicity (Chen et al., 1994). This makes licochalcone A **50**, a candidate for further

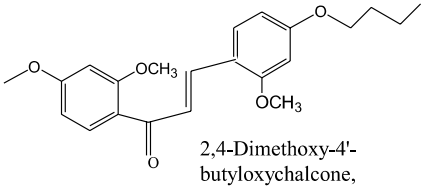
Chalcones



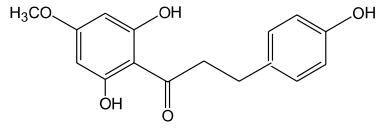
Uvaretin, **49**



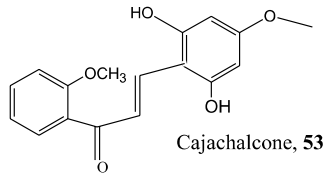
Lichochalcone, **50**



2,4-Dimethoxy-4'-butyloxychalcone, **51**



Asobogenin, **52**



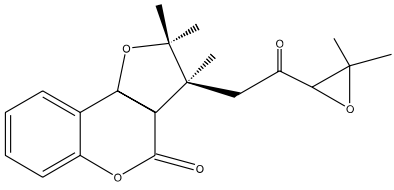
Cajachalcone, **53**

development, being active in the *in vitro* assays, and not toxic even at such a high dose in the animal studies using the *Plasmodium* sp. in mice. These promising results led to synthesis of various chalcone derivatives. Chen et al. (1997) studied the antimalarial properties of 2,4-dimethoxy-4-butyloxychalcone **51**, a synthetic chalcone derivative, using *P. falciparum* for *in vitro* assay and *P. berghei* and *P. yoelii* for studies in mice. *Piper hispidum* (Piperaceae) furnished asobogenin **52** as the antimalarial compound (Jenett-Siems et al., 1999). From the Nigerian antimalarial phytomedicine, a chalcone; cajachalcone **53**, isolated from *Cajanus cajan* (Fabaceae) has displayed antiplasmodial properties in the parasite lactate dehydrogenase assay, using multiresistant strain of *P. falciparum* K1 with an IC_{50} of 2.0 µg/ml (Ajaiyeoba et al., 2003, 2005).

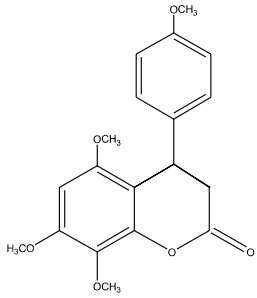
Coumarins—Oketch-Rabah et al. (1997) reported the presence of two coumarins; 2-epicycloisobrachycoumarinone epoxide **54** and cycloisobrachycoumarinone epoxide **55** as the active compounds from the roots of the East African medicinal plant, *Vernonia brachycalyx* (Asteraceae). Both compounds were more active against the chloroquine-resistant parasite Dd2 strain, which was used in the study, with IC_{50} of 54 µM respectively, compared to 111-160 µM for the chloroquine-sensitive strain, 3D7.

Several coumarins were identified from *Exostema mexicana* (Rubiaceae) stem bark and were subjected to *in vitro* antimalarial studies. The most

Coumarins



Epicycloisobrachycoumarinone, **54** - R^1=CH_3, R^2 =H
Cycloisobrachycourmarinone, **55** - R^1=H, R^2=CH_3

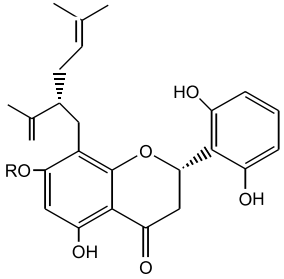


O-Methylexostemin, **56**

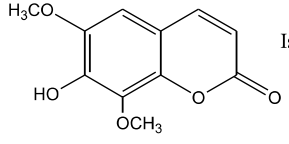
active of the coumarin was O-methyl exostimin **56**. It had an IC_{50} value of 3.6 µg/ml against the chloroquine-sensitive strain of *P. falciparum* and an IC_{50} value of 1.6 µg/ml against the chloroquine resistant strain (Kohler et al., 2001).

Flavones and Flavonoids—The detection of antiplasmodial **flavonoids** in *Artemisia annua* (Asteraceae) led to the study of this class of compounds with renewed interest. Two methoxylated flavones, artemetin and casticin act synergistically with artemisinin **2**, against *P. falciparum*in in the *in vitro* analysis. None of the other compounds were as active as artemisinin. Further examination of *Artemisia* species led to the identification of more antiparasitic flavonoids. One of such studies in the Thai ethnomedicine furnished exiguaflavanone A **57** and exiguaflavanone B **58** from *A. indica*. Both compounds exhibited activity with EC_{50} values of 4.6 and 7.1 µg/ml respectively, in the *in vitro* analysis using *P. falciparum* (Chaphen et al. 1998). From *A. arbrotanum*, isofraxidin **59** was identified as the antimalarial component (Cubucku et al., 1990). The isoflavones, calycosin **60** and genistein **61** isolated from *Andira inermis* (Fabaceae) have been shown to have antimalarial properties.

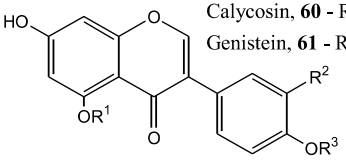
Flavones and Flavanoids



Exiguaflavanone A, **57** - R=CH_3
Exiguaflavanone B, **58** - R=H



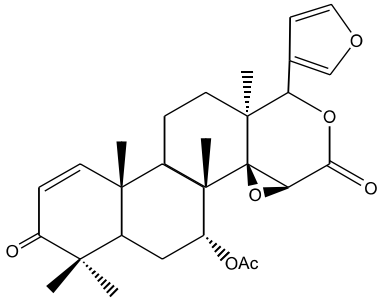
Isofraxidin, **59**



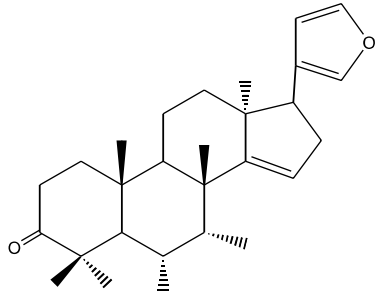
Calycosin, **60** - R^1=H, R^2=OH, R^3=CH_3
Genistein, **61** - R^1=OH, R^2, R^3=H

Limonoids—Practically all the plants belonging to the family Meliaceae have been investigated for antimalarial properties because of the use of these plants in several ethnomedicines in Africa, Asia, and South America for malaria therapy. Over 60 extracts from various parts of over 20 plants were tested against *P. falciparum*. Of particular interest in this regard is the Neem tree, *Azadirachta indica* (Meliaceae), which has undergone various antiparasitic studies. The antimalarial activity of *A. indica* has been attributed to the presence of gedunin **62** (MacKinnon et al., 1997). In a previous study, Khalid et al. (1986) reported that **62** add effective concentration, isolated from *Melia azedarach* had activity comparable to quinine in the *in vitro* assay using *P. falciparum* and ^{3}H- hypoxanthine in continuous culture**.** The *in vivo* mice model activity of this compound using *P. berghei* was not promising for further development (Bray et al. 1990). From the Indian ethnomedicine, meldenin **63** was the most active of four limonoids isolated from in a bioassay-guided isolation of *A. indica* leaves. Azadirachtin **64** and three semisynthetic limonoid derivatives were found to be active against the erythrocytic stages of *P. falciparum*. The neem extracts were reported to affect all the stages of maturation of the gametocytes. This

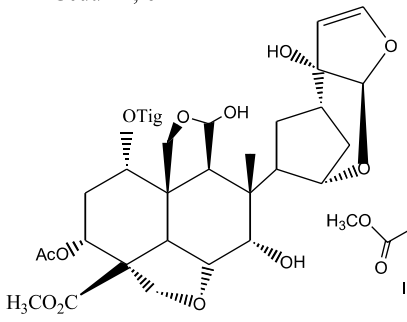
Limonoids



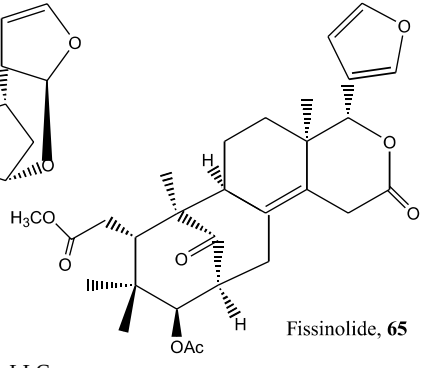
Gedunin, **62**



Meldenin, **63**



Azadirachtin, **64**

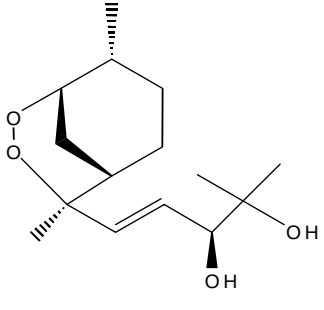


Fissinolide, **65**

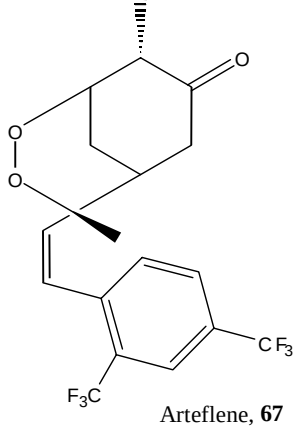
was novel and unlike the reference drugs artemisinin **2** and primaquine that inhibit only the immature stages of growth (Dhar et al., 1998). From other Meliaceae plants, most especially the genus *Khaya* had provided antiplasmodial agents. Fissinolide **65** from *K. senegalensis* was active against chloroquine-resistant *P. falciparum*. The non-polar extract from *K. grandifololia* was found active in the *in vivo* antiplasmodial analysis using *P. berghei* in mice (Khalid et al., 1998; Agbedahunsi et al., 1998).

Peroxides—Ever since the artemisinin **2**, a macrocylic sesquiterpene endoperoxide, from *A. annua* and its analogs have been shown to possess great promise as rapidly acting and potent antimalarials, a lot of attention has been focused on various natural peroxides and several have been reported to display antimalarial properties (Klayman, 1985; Dhingra et al., 2000). *Artemisia annua* (Asteraceae) is the Chinese herb that had been used in the Chinese ethnomedicine for over three centuries for the treatment of malaria. Presently, artemisinin and its derivatives are by far the most useful compounds for treatment of chloroquine-resistant malaria. Though several synthesis of artemisinin has been reported, the most cost-effective means of obtaining the compound is still through the plant (van Agtamael et al., 1999). The peroxide yingzhaosu **66,** also from the Chinese ethnomedical plant *Artabotrys uncinatus* (Annonaceae), used for malaria treatment was reported to have exceptionally good activity. This initiated the synthesis of arteflene **67**, with a high potential as a candidate worthy of development. Ascaridole **68**, a terpene endoperoxide isolated from *Chenopodium ambrosiodes* (Chenopodaceae) has been shown to possess antiplamodial properties (Pollack et al., 1989). The bisabolene endoperoxides zingiberene 3, 6-α- and β-endoperoxides **69,** identified from the Brazilian plants *Eupatorium rufescens* and *Senecio selloi* (Asteraceae) were found to display

Peroxides

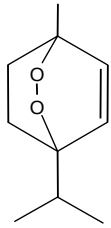


Yingzhaosu, **66**

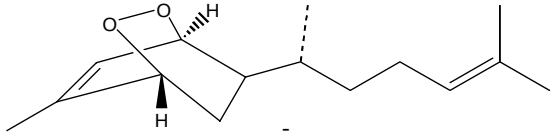


Arteflene, **67**

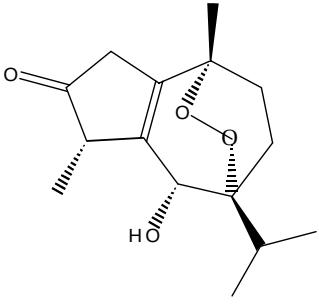
Peroxides (continued)



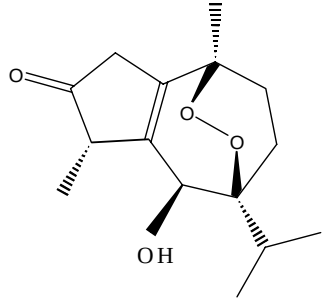
Ascaridole, **68**



Zingiberene 3,6-ß-endoperoxide, **69**



Nardoperoxide, **70**

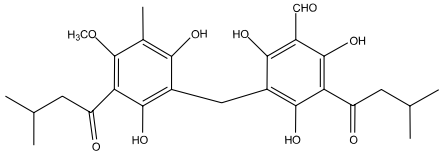


Isonardoperoxide, **71**

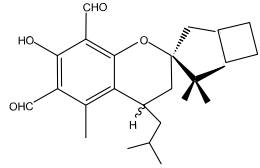
antimalarial properties. The sesquiterpene endoperoxide, 10,12-peroxy-calamenene was found to be the most active compound in the *in vitro* bioassay-guided isolation of *Cyperus rotundus*, the Tanzanian and Thai antimalarial plant (Weenen et al., 1990). Nardoperoxide **70** and isonardoperoxide **71** were isolated from the roots of *Nardostachys chinensis* and they had EC_{50} of 1.5 µM and 0.6 µM respectively in the *in vitro* studies using *P. falciparum* (Takaya et al., 1998).

Phloroglucinol compounds—This group of compounds have shown a wide range of biological activities including antimalarial and anti HIV properties. Phloroglucinol compounds occur widely in *Eucalpytus sp* and *Hypericum sp*. The leaves of *E. robusta* (Myritaceae) used in China for treatment of malaria among other ailments, furnished robustaol A **72**, robustadial A **73** and B **74** as the antimalarial compounds (Lopes et al., 1999). The relative prenylated stilbene derivatives; **(75, 76** and **77**) from the fruit and aerial parts of *Artrocarpus interger* (Moraceae) showed antimalarial activity (Boonlaksiri et al., 2000). Sarothralen B **78** and Japonicine A **79**, from *Hypericum japonicum* (Clusiaceae) and the prenylated phloroglucinol derivative **80** from *H. calycinum* were found to possess good antimalarial activity (Xu et al., 1984).

Phloroglucinol Compounds

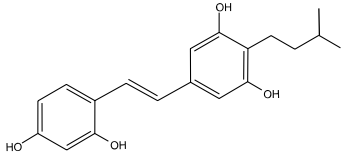


Robustaol A, **72**

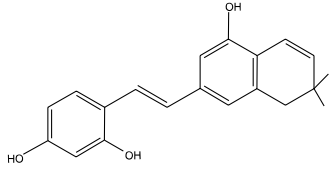


H-7α–Robustadial A, **73**

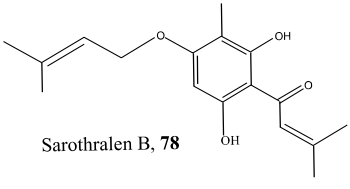
H-7β–Robustadial B, **74**



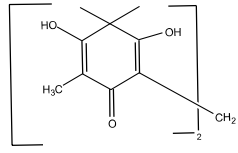
1", 2" - Dehydro, Stilbene derivative 1, **75**
2", 3" - Dehydro, Stilbene derivative 2, **76**



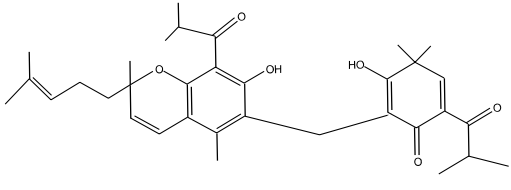
Stilbene derivative 3, **77**



Sarothralen B, **78**



Japonincine A, **79**

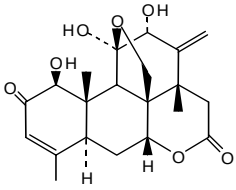


Phloroglinol derivative, **80**

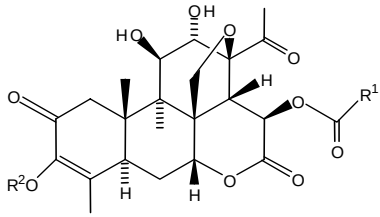
Quassinoids—These are bitter principles obtained primarily from the Simaroubaceae plant family. They are active principles that have been reported to demonstrate cytotoxicity and antimalarial properties. Antimalarial and cytotoxicity results have been shown not to correlate, indicating antimalarial selectivity. Extracts and isolated compounds from seedlings of *Ailanthus altissima*, were assessed for antiplasmodial activity *in vitro* (Bray et al., 1987). Two quassinoids, ailanthone **81** and 6α-tigloyloxychaparrinone, isolated from the active extracts showed activity against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* in the *in vitro* analysis. Only ailanthone **81**

demonstrated low toxicity against the Vero cell line (Okuande et al., 2003). *Brucea javanica* was one of the foremost quassinoid-containing plants from the Simaroubaceae plant family to be evaluated for antimalarial activity. In Thai and Chinese ethnomedicines, *B. javanica* is used in the treatment of malaria usually as a tea in combination with chloroquine. Bruceine A **82**, bruceine B **83** and bruceine C **84** isolated from the chloroform fraction of the fruit of the plant displayed activity against multi drug-resistant strain of *P. falciparum* comparable to mefloquine (Pavanand et al., 1988) Add effective concentration. Yandanziolide **85** of the 12 quassinoids isolated by O′ Neill et al. (1987) from the same plant was found most active and was even more so than chloroquine salt used in the study. Compounds **82-84**, brustanol **86** and bruceine D **87** were active in the mice model antimalarial study using *P. berghei*. Though these compounds were toxic and reported to be antagonistic to chloroquine, they may contribute to developing chloroquine - resistant malaria chemotherapeutic agents. *Brucea sumatrana* also furnished bruceolide **88** and this demonstrated activity against *in vivo* using *P. berghei* in mice. Dou et al. (1996), identified seven active quassinoids from *Castela texana* which demonstrated *in vitro* antiplasmodial properties using both the chloroquine sensitive and resistant strains of *P. falciparum*, the most active of which was holacanthone **89** with IC_{50} of 0.010 µg/ml. From Malaysian *Eurycoma longifolia* roots, eurycomanol 2-O-glucopyranoside **90** was found to be very active on chloroquine resistant strain of *P. falciparum* with an IC_{50} of 0.39-3.50 µg/ml (Ang et al., 1995). Of the four quassinoids isolated from the stem bark of *Hannoa chlorantha* and *Quassia undulata* (formerly, *H. klaineana*), Chapparinone **91**, displayed a good activity in the *in vitro* studies using the NF54, clone AIA9 of *P. falciparum*. It also exhibited antimalarial properties in the mice model study with the Anka strain of mice (Francois et al., 1998). *Quassia undulata* and *Q. amara* extracts also displayed antimalarial activities in mice using Anka clones of *P. berghei*. At a concentration of 100 mg/kg mouse, *Q. amara* leaf methanol extract had the highest suppressive activity with a parasite density of 0.16% and chloroquine had a parasite density of 0.34% in the same assay on day 4 (Ajaiyeoba et al., 1999). *Quassia indica* furnished antiplasmodial compounds, the samaderine X, samaderine Z, samaderine B and samaderine E **92** and which were active against chloroquine resistant (K1) strain of *P. falciparum* with an IC_{50} of 0.14-0.21 µM (Kitagawa et al., 1996a). Gutolactone **93** and simalikalactone D **94** from *Simaba quaianesis* and *Q. africana*, have demonstrated interesting antimalarial and antiviral activities (Apers et al., 2002). Both compounds have demonstrated the highest activities of all the quassinoids with IC_{50} of 4.0 and 1.6 ng/ml each. In the same assay, chloroquine and mefloquine had IC_{50} values of 63.2 and 1.5 ng/ml, respectively. Moretti et al. (1998) identified cedronin **95** from *Simana*

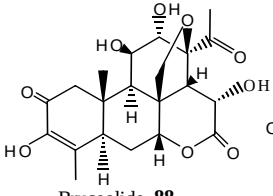
Quassinoids



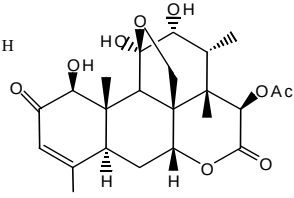
Ailanthone, **81**



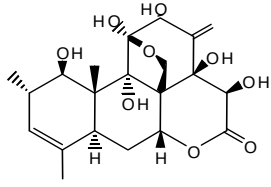
Bruceine A, **82** R^1 = Isobutyl, R^2 = H; Bruceine B, **83** R^1 = CH_3, R^2 = H
Bruceine C, **84** R^1 = $CH{=}C(CH_3)C(OH)(CH_3)_2$, R^2 = H;
Yadanziolide I, **85**,R^1 = CH_3, R^2 = glucopyranoside;
Brustanol **86**, R^1 = $CH{=}C(CH_3)_2$, R^2 = H; Bruceine D, **87** R^1 = H, R^2 = CH_3



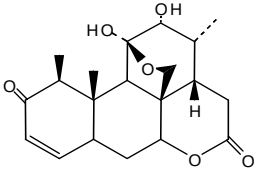
Bruceolide, **88**



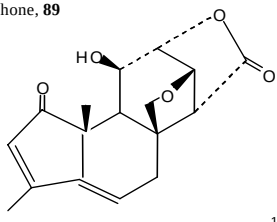
Holacatnhone, **89**



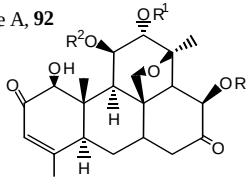
Eurycomanol glucoside, **90**



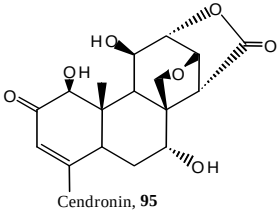
Chapparinone, 91



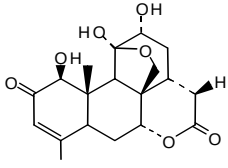
Samaderine A, **92**



Gutalactone, **93**- R = Me_2 C=CHCO, R^1 ,R^2 = H
Simakilactone D, **94** = Me $CH_2CH(Me)CO$, R^1 , R^2 =Ac



Cendronin, **95**



Quassinoid C07D493-08, **96**

cendron and it showed a much better activity against both chloroquine sensitive and resistant strains of *P. falciparum* in the *in vitro* investigation as well as *in vivo* study using *P. vinckei petteri* in mice. A quassinoid registered under CO7D493-08, **96** in the patent registry, with a structure similar to cendronin was reported much earlier, to increase the survival time in male mice, when innoculated with *P. berghei* NK-65 compared with

11.3 days for bruceantin (Takenuchi, 1989). The presence of an ester group at C-15 has been reported to be vital for activity and the glycosides were generally less active than the aglycones (Bray et al., 1987). Table 4 below displays the antiplasmodial properties of some compounds in Simaroubaceae family.

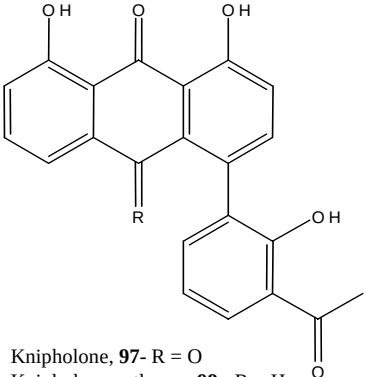
Table 4. Antiplasmodial properties of quassinoids of the simaroubaceae plant family

*Compound (**Figure**)*	*Plant source*	*P. falciparum in vitro activity/ strain (IC_{50} µg/ml)*		
		D_6	W_2	*K1 strain**
Holacanthone (**90**)	*Castela texana*	0.010	0.120	
Eurycomanol glucoside (**91**)	*Eurycoma longifolia*	–	0.420	
Chapparinone **(92)**	*Quassia undulata*	0.250	0.20	
Samaderine E (**95**)	*Q. indica*	–	–	0.056 [a]
Gutolactone (**97**)	*Q. africana/Simaba quaianensis*	–	–	0.004
Simikalactone D (**98**)	*Q. africana/Simaba quaianensis*	-	-	0.002

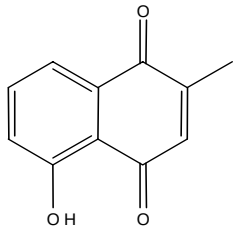
*D_6 = Chloroquine sensitive; W_2 = Chloroquine resistant; K1 = multi resistant
a = IC_{50} in µM.

Quinones—*Bulbine capitata* (Asphodelaceae) from the Botswana ethnomedicine furnished 5 naphthoquinone furan diones with 1-acetoxymethyl-8-hydroxynaphtho(2,3-c)furan-4,9 dione showing the highest activity against chloroquine sensitive (3D7, IC_{50} of 23 µM) and chloroquine resistant (K1, IC_{50} of 52 µM) strains of *P. falciparum* (Bezabih et al., 2001). From the roots and aerial parts of *B. capitata,* knipholone **97**, 4'-O-demethylknipholone and 6'-O-methylknipholone were identified and isolated. Demethyl knipholone also exhibited the highest activity against chloroquine sensitive (NF54, IC_{50} of 1.55 µM) while knipholone **97** was more active against the chloroquine resistant K1 strain of *P. falciparum* with an IC_{50} of 1.06 µM. Knipholone anthrone **98** was also identified as a very active quinone from *Knipholia foliosa* and was more active than the other quinones (IC_{50} of 0.31 µM) with the chloroquine resistant parasite (Bringmann et al., 1999). Plumbagin **99**, identified in *Nephenthes thorelli* (Nepethenceae) was the most active of the five naphthoquinones against *P. falciparum* with an IC_{50} of 0.27 µM (Likhitwitayawuid et al., 1998a). The antimalarial properties of lapachacol **100**, identified from several Bignoniaceae plants initiated the synthesis of the antimalarial drug, atovaquine **101**. Isopinnatal **102** isolated from *Kigelia pinnata* was also

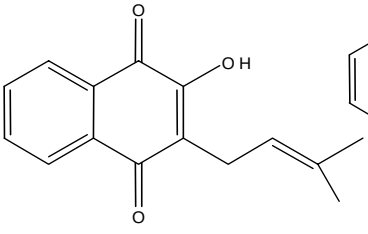
Quinones



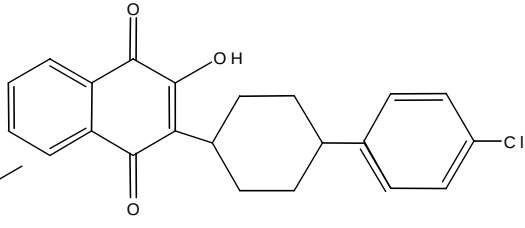
Knipholone, **97**- R = O
Knipholone anthrone, **98** - R = H_2



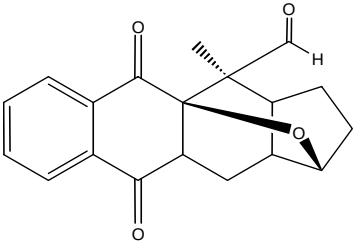
Plumbagin, **99**



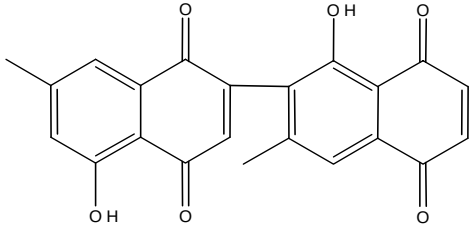
Lapachacol, **100**



Atovaquine, **101**



Isopinnatal, **102**

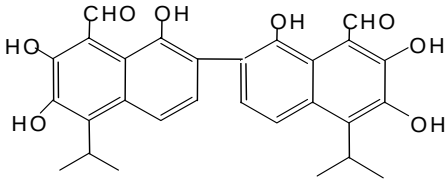


Diosporin, **103**

found to display antimalarial properties. *Diospyros montana* (Ebeneceae) has furnished diosporin **103** with a reported in vitro activity against *P. falciparum* (Hazra et al., 1995).

Tannins—The biggest drawback in the development of tannins as an antimalarial drug has been lack of selectivity with malarial parasite as compared to cancer cells, though tannins have displayed antiplasmodial properties. Gossypol **104**, isolated from species of the cotton seed plant,

Tannins

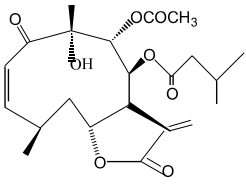


Gossypol, **104**

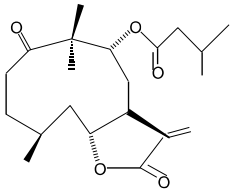
Gossypium, has demonstrated impressive antiplasmodial properties. It was shown to inhibit chloroquine-sensitive and chloroquine-resistant *P. falciparum* parasites with IC_{50} in the magnitude of 10 μM. It has also been shown to be cytotoxic (Coyle et al., 1994; Deck et al., 1998). The traditional healers in Southwest Nigeria identified *Gossypium arboreum* leaves as the most potent of the three species including *G. hirsitum* and *G. barbadense* plant for treatment of febrile illness. Though the crude methanol extracts elicited weak activities (IC_{50} = 197.9 μg/ml) with the multi-resistant strain of *P. falciparum* K1 strain, in the clinical observation study, a *P. falciparum* parasite clearance of over 70% was recorded in febrile clients of the herbalists. In the *in vitro* analysis using KB cells, the crude methanol extract of the leaves was also found to be non-toxic with ED_{50} > 300 μg/ml (Ajaiyeoba et al., 2004a, 2004b).

Terpenoids—Following the discovery and subsequent development of the sesquiterpene endoperoxide, Artemisinin **2**, from *Artemisia annua*, several sesquiterpenoids mainly from the Asteraceae plant family and indeed most terpenoids have been evaluated for antimalarial properties (Trigg, 1989). From the Central American phytomedicinal plant, *Neurolaena lobata*, several antiplasmodial sesquiterpenoids have been isolated. The three most active compounds being Neurolenin B, **105**, Neurolenin C **106** (each with IC_{50} of 0.62 μM), using the chloroquine sensitive *P. falciparum* NF54 strain (Francois et al., 1996). From another Asteraceous plant, Takaya et al. (1998) isolated the sesquiterpenoids, Nardoperoxides **83** and isonardoperoxide **84** (as previously discussed), among others from the roots of *Nardostachys chinensis* (Asteraceae). The Kenyan plant *Vernonia brachycalyx* (Asteraceae) furnished the sesquiterpenoid 16,17 Dihydrobrachycalyxolide, **107** which was studied for antimalarial properties using several *P. falciparum* strains. It was most sensitive to the chloroquine-resistant *P. falciparum* V1/S strain and in the bioassay; it had an IC_{50} value of 5.9 μM (Oketch-Rabah et al., 1998).

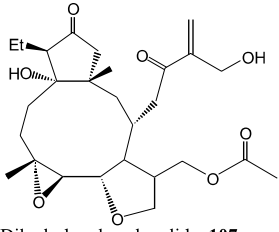
From Surinam, Lopes et al. (1999) identified nerolidol as one of the antimalarial components in *Virola surinamensis*. An antimalarial eudesmane



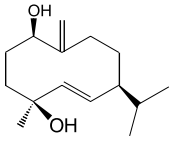
Neurolenin B, **105**



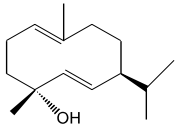
Neurolenin C, **106**



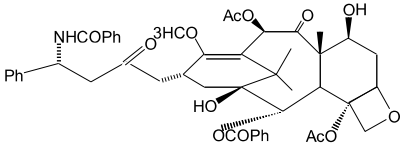
16,17-Dihydrobrachycalyxolide, **107**



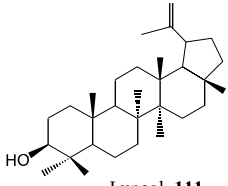
Reneilmia Sesquiterpenoid A, **108**



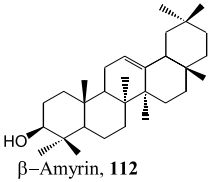
Reneilmia Sesquiterpenoid B, **109**



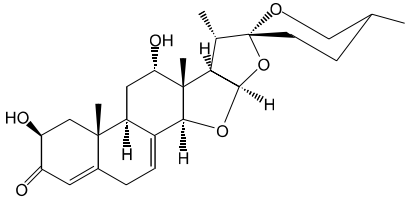
Taxol, **110**



Lupeol, **111**



β–Amyrin, **112**



Muzazangenin, **113**

sesquiterpenoid has also been reported from *Jasonia glutinosa* (Villaescusa-Castillo et al., 2000). The Cameroonian ethnomedicine furnished the two antiplasmodial sesquiterpenoids **108** and **109** from *Reneilma cincinnata*. Their IC_{50} values renged from 1.5-1.6 µg/ml for the chloroquine-sensitive strain, D6 and between 1.9-31.9 µg/ml for the resistant strain W2 (Tchuendem et al., 1999).

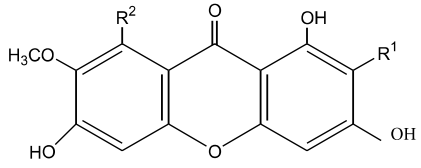
The diterpenoid, taxol **110**, from the Pacific yew, *Taxus brevifolia* an anticancer agent was shown by Pouvelle et al. (1994) to display antimalarial properties *in vitro* using *P. falciparum* and *in vivo* with *P. chabaudi adami* in mice.

From *Vernonia brasiliana* (Asteraceae), Lupeol **111** and β-amyrin **112**

were identified as the antiplasmodial compounds (de Almeida-Alves et al., 1997). Lupeol was also identified from the Nigerian phytomedicine as the antimalarial compound from *Cassia siamea* and *C. nigricans* (Fabaceae) respectively with an IC_{50} of 5.0 µg/ml using the multi-resistant strain K1 of *P. falciparum* (Ajaiyeoba et al., 2007; Obodozie et al., 2004). Betunilic acid isolated and identified from several plant species has shown weak antiplasmodial properties using different *P. falciparum* strains. Steele et al. (1999) and Traore-Keita et al. (2000a) reported that it had IC_{50} of 19.6 µg/ml (multi-resistant K1 strain) and 18.10 µg/ml (resistant W2 strain). Muzanzagenin **113** was identified by Oketch-Rabah et al. (1997) as the antimalarial triterpenoid from the Kenyan medicinal plant, *Asparagus africanus* (Liliaceae). The diterpenoid, taxol **114**, from the Pacific yew, *Taxus brevifolia* an anticancer agent was shown by Pouvelle et al. (1994) to display antimalarial properties *in vitro* using *P. falciparum* and *in vivo* with *P. chabaudi adami* in mice. The leaf extract of *Hyptis suaveolens* (Lamiaceae), an abietane type diterpenoid endoperoxide **115**, was identified as the antiplasmodial agent with an IC_{50} of 4.1 µg/ml using a chloroquine sensitive strain of *P. falciparum*, D10 (Chukwujekwu et al., 2005). From *Vernonia brasiliana* (Asteraceae), Lupeol **116** and β-amyrin **117** were identified as the antiplasmodial compounds (de Almeida Alves et al., 1997). Lupeol was also identified from the Nigerian phytomedicine as the antimalarial compound from *Cassia siamea* and *C. nigricans* (Fabaceae) respectively with an IC_{50} of 5.0 µg/ml using the multi-resistant strain K1 of *P. falciparum* (Obodozie et al., 2004; Ajaiyeoba et al., 2007). Betunilic acid isolated identified from several plant species has shown weak antiplasmodial properties using different *P. falciparum* strains. Steele et al. (1999) and Traore-Keita et al. (2000) reported that it had IC_{50} of 19.6 µg/ml (multi-resistant K1 strain) and 18.10 µg/ml (resistant W2 strain). Muzanzagenin **118** was identified by Oketch-Rabah and Dossaji (1997) as the antimalarial triterpenoid from the Kenyan medicinal plant, *Asparagus africanus* (Liliaceae).

Xanthones—Species of *Garcinia* have furnished several xanthones that have been active in antimalarial assays. Of the five xanthones isolated from *G. cowa* (Guttiferae), Cowaxanthone **119** and Cowanol **120** were the most impressive with IC_{50} of 1.5 µg/ml, and 1.6 µg/ml, respectively in the study with *P. falciparum in vitro* (Likhitwitayawuid et al., 1998a). Five xanthones were also isolated from *G. dulcis* and the most active antimalarial compound was found to be garciniaxanthone **121** (Likhitwitayawuid et al., 1998b; Wright et al., 2000). Dua et al. (2004) identified six xanthones from the leaves of *Andrographis paniculata*, a popular ethnomedicinal plant in China and Southeast Asia. *In vitro* antimalarial studies showed that 1,2-dihydroxy-6,8-dimethoxy xanthene-9-one (TDR 130011), **122** exhibited the highest activity with an IC_{50} of 4 µg/ml with *P. falciparum* and gave a

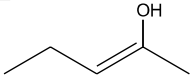
Xanthones



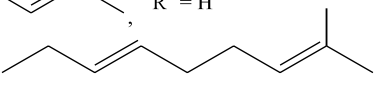
Cowaxanthone, **114** - R^1 =

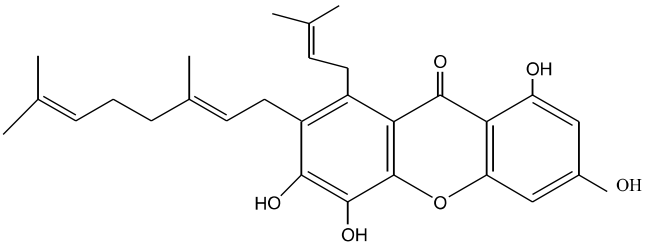
, R^2 = H

Cowanol, **115** - R^1 =

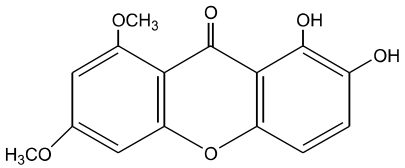


, R^2 =





Garciniaxanthone, **116**

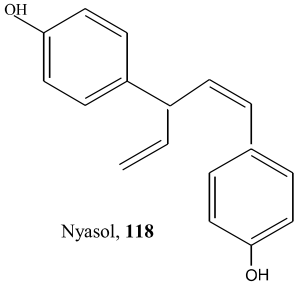


TDR 130011 Xanthene-9-one, **117**

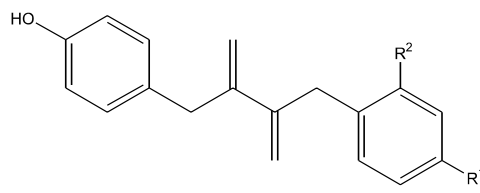
70% reduction using *P. berghei* in mice without showing toxicity in the human lung fibroblast (Dou et al., 1999).

Other compounds—The lignan nysol **123** from *Asparagus africanus* (Liliaceae) was also shown to possess antimalarial properties *in vitro* and *in vivo* mice model (Oketch-Rabah et al., 1997). *Termenalia bellerica* (Combretaceae) fruit rind from the Indian ethnopharmacology has presented termilignan **124** and anonlignan **125** as antiplasmodial compounds. The two most active lignans isolated from *Rhaphidophora decursiva* (Araceae) leaves and stems were polysyphorin **126** and rhaphidecurperoxin **127**. Polysyphorin, **128** had an IC_{50} value of 0.040 µg/

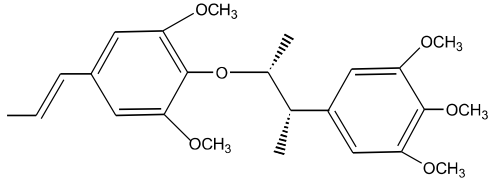
Other Compounds



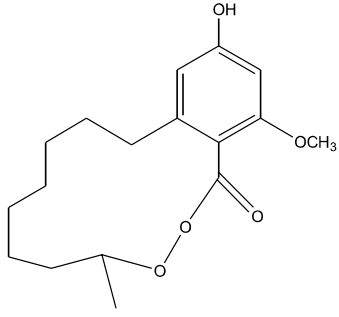
Nyasol, **118**



Anolignan, **119** - $R^1 = OH$, $R^2 = H$
Termilignan, **120** - $R^1 = OCH_3$, $R^2 = OH$



Polysyphorin, **121**



Rhadphidecurperoxin, **122**

ml against the chloroquine-sensitive strain and 0.037 µg/ml (3D7) with the chloroquine resistant strain (Dd2), used in the study (Zhang et al., 2001).

Future perspectives in chemotherapeutic control of malaria

Malaria is a major health burden in developing countries and is a remaining health problem socio-economically in developed countries; it is important that various strategies and interventions are planned and executed. Malaria chemotherapy is even more complex and challenging

because of multi-drug resistant strains of *Plasmodium falciparum*, which are readily available and well known chemotherapeutic agents are now ineffective. There is no doubt that the new drugs against malaria are greatly needed (Ridley, 2002). Rosenthal (2003) in his review of old and new approaches of antimalarial drug discovery highlighted the under-listed strategies: discovery of natural products, development of analogs of existing chemotherapeutic agents evaluation of other compounds used previously for other diseases, evaluation of drug resistant reversers and consideration of new drug targets. These are basically the five practical areas that should constitute the focus in malaria drug discovery and development for the future.

Presently, no vaccines are available for malarial treatment and the prospect for vaccine development is not soon enough, hence drugs will remain the main stay of disease control (WHO, 2004). Since the two most effective drugs for control of malaria originate from plants, that is quinine **1** and artemisinin **2**, plants are a major resource for discovering of newer malaria drugs. The screening of herbs using local biodiversities should be enhanced (Wilcox and Bodeker, 2000). Approaches to discovery of antimalarial agents from various biodiversities are not different from those for other diseases. These could be through:

1. Ethnomedicinal leads
2. Random screening
3. Literature
4. Chemotaxonomy

These are the four basic approaches used in the selection of plants. Thereafter, selected plants are primarily subjected to *in vitro* antiplasmodial screens, to identify a 'hit'. This refers to that plant sample/drug that has an index of inhibitory activity greater than the reference drug utilized in the same assay. *In vivo* animal screens mainly in murine animals are regarded as the secondary screens. This also helps in identifying plants that contain constituents that may be prodrugs. Isolation, mainly by a combination of chromatographic techniques and subsequent spectroscopic structural elucidations of isolated bioactive components usually follow. Structure-activity relationships may be initiated to maximize observed bioactivity or reduce toxicity to optimize lead antimalarial compound(s). Various stages of toxicological assessments in animal models will be necessary for the selected compound to progress to the next stage, which usually precede biopharmaceutical studies (pharmacokinetics, bio-availability properties of the lead compound). Other stages of drug development generally will include other tertiary bioassays and studies including the possibility of synthesis of the compound. By so doing, in a couple of years, usually more than 10 years, a development candidate emerges.

In most developing countries where malaria is endemic, it is reported that over 80% of the rural population with no access to modern healthcare facilities, depend on traditional medicine for management of most diseases including malaria. In the short term, the use of herbal remedies consisting of plants like the neem tree, *Azadirachta indica* (Meliaceae), *Khaya senegalensis* (Meliaceae), *Morinda lucida* (Rubiaceae), *Cassia* sp. (Fabaceae), *Phyllanthus* sp. (Euphorbiaceae), to mention a few, has been reported (Ekanem, 1978; Makinde et al., 1994; Agbedahunsi et al., 1998; Ajaiyeoba, 2002; Ajaiyeoba et al., 2002, 2003, 2004, 2005, 2006, 2007) from Nigeria. Investigations on some of the individual plants have indicated some antiplasmodial properties, both in the primary *in vitro* and *in vivo* assays. This has assisted the indigenous communities in the management of uncomplicated malaria, while awaiting drug development from hit plants from these ethnomedicines, as in the case of the Peruvian and Chinese ethnomedicines that provide the two major drugs for chemotherapeutic treatment of malaria; quinine **1** and artemisinin **2**, respectively.

Conclusion

Malaria still poses the greatest threat of all parasites to human health. Malaria is a major health problem and its eradication has so far proved impossible. The eradication of malaria seems to be ampiclox, mainly due to resistance of the parasite to readily available drugs and so loss of efficacy. The death toll from malaria especially in Africa is likely to be on the increase with the spread of drug resistance to chloroquine. This has made the chemotherapy of malaria shift to drug combination and a re-visitation of abandoned drugs. Vaccine development is still being awaited. So wither hence? The future of chemotherapy as the mainstay for malaria may look clouded. In this article, a large number of compounds with structurally diverse chemical structures have been mentioned, there is no doubt that these plant secondary metabolites present a plethora of compounds that could be developed into the new antimalarial drugs. Ethnopharmacological approach has by far proved superior to other empirical approaches in identification of new sources of antimalarial drug. Hence scientific validation of traditional medicinal properties of indigenous medicinal plants used for management of malaria should be encouraged. A concerted effort by all stakeholders in this regard, may well provide the answer for the immediate control of malaria in sub-Saharan Africa and other endemic regions. Nature has continued to provide solutions to problems of people on earth and chemotherapeutic malaria control is one of the problems that can be overcome from natural sources.

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5 Anti-*Candida* Activity of Extracts and Essential Oils from Native and Exotic Medicinal Plants in Brazil

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Introduction

Drug-resistance to human and animal microbial pathogens is one of the best-documented cases of biological evolution and a serious problem in developing as well as developed countries. More than one tonne of antibiotics per day are consumed in some European countries, which has resulted in the emergence and spread of a vast amount of antibiotic resistance determinants among bacterial populations, thus creating a critical public health problem. Baquero and Blázquez (1997) stated that the danger of a return to a pre-antibiotic era is becoming a serious threat, particularly considering that no novel chemical class of antibiotics has been introduced in the past 20 years, despite intensive research in the pharmaceutical industry. In view of the present scenario, the search for new antimicrobial substances from natural sources including plants, is gaining importance for pharmaceutical companies.

The use of plants as a source of medicine is prevalent in developing countries as an alternative solution to health problems, and is well established in some cultures and traditions, especially in Asia, Latin America and Africa (Shale et al., 1999). Many of these plants have not been studied, and can be researched for antimicrobial action, in contrast to European native plants that have already been exhaustively studied. Phytotherapy was adopted more in the past mainly by a needy population

from rural or urban areas, due to easy availability and cheaper costs. However these days due to the increasing interest in natural products, the usage of herbal medicine has became more or less, general.

History has recorded several accounts on plant utilization for treatment of diseases since 4,000 B.C. The first medical report deposited at the Pennsylvania Museum dates from 2,100 B.C and includes a formula collection of 30 different drugs arising from plants, animals and minerals (Helfand and Cowen, 1990). The Egyptian manuscript 'Ebers Papirus' (1.500 a.C.) contains 811 prescriptions and 700 drugs, and the first Chinese text on medicinal plants (500 a.C.) mentions names, dosage and indications of plants used for the treatment of diseases, which are still used such as Ginseng (*Panax* spp.), *Ephedra* spp., *Cassia* spp and *Rheum palmatum* L. Some of them are sources for pharmaceutical industries.

Though antimicrobial properties of substances present in extracts and essential oils which plants produce as a consequence of secondary metabolism have been recognized empirically over centuries, but were scientifically confirmed only recently (Jansen et al., 1987). Studies on the antimicrobial activities of the extracts and essential oils of native plants have been reported in many countries such as Brazil, Cuba, India, Mexico and Jordan, which are examples of countries that have diverse flora and a rich tradition in the use of medicinal plants for both antibacterial and antifungal applications (Martínez et al., 1996; Navarro et al., 1996; Ahmad and Beg, 2001; Mahasneh et al., 1999; Rehder et al., 2004).

Since medicinal plants produce a variety of compounds with antimicrobial properties, it is expected that screening programs for some under-represented targets, such as antifungal activity, may yield candidate compounds for developing new antimicrobial drugs (Ahmad and Beg, 2001). However, scientific investigation in order to determine the therapeutic potential of the plants is limited and there is still a lack of experimental scientific studies confirming the possible antibiotic properties of a great number of these herbs. It is expected that plant compounds showing target sites other than those currently used by antibiotics will be active against drug-resistant microbial pathogens.

Natural products and anti-*Candida* research around the world

Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries; dermatophytes and *Candida* spp. being the most frequent pathogens (Portillo et al., 2001). *Candida albicans* is an opportunistic pathogen 'yeast' that can cause local or systemic infections in predisposed persons, commonly affecting immunologically compromised

patients and those undergoing prolonged antibiotic treatment (Zhang et al., 2002a). However, information available on medicinal plants active against this yeast species has, until recently, not resulted in effective formulations for humans or animal use, except for some patents from material derived from the plant family *Allium* (Plummer, 1992), from *Radix gentianae* (Chen, 1996) and five extracts studied by Lee et al. (2003). Recently, several research groups from different countries have studied the inhibition of *C. albicans* by extracts, essential oils or isolated substances from plant groups. In many countries such as India and from Africa and Latin America, most of the work began from an ethnopharmacological survey, which describe the species most frequently used by the population. Some Latin American countries have maintained research programs to screen traditional medicines for antimicrobial activity, as is the case of Cuba (Martínez et al., 1996), Honduras (Lentz et al., 1998) and Mexico (Navarro et al., 1996; Rojas et al., 2001).

The investigation of natural products active against *Candida* spp. around the world increased significantly since 2003 as can be seen from Figure 1, which shows the number of indexed papers (ISI) in the last 14 years. This increase was higher in Latin America, Europe and Asia (Figure 2). Despite many studies, the present survey does not consider results on local divulgations such as meeting abstracts and academic degree theses that are generally not published.

Among the investigated species, Table 1 shows only medicinal plants that presented positive activity against *C. albicans*, as well as the plant part used and type of sample preparation. The data presented in Table 1 apparently indicates that a significant number of plant families and species

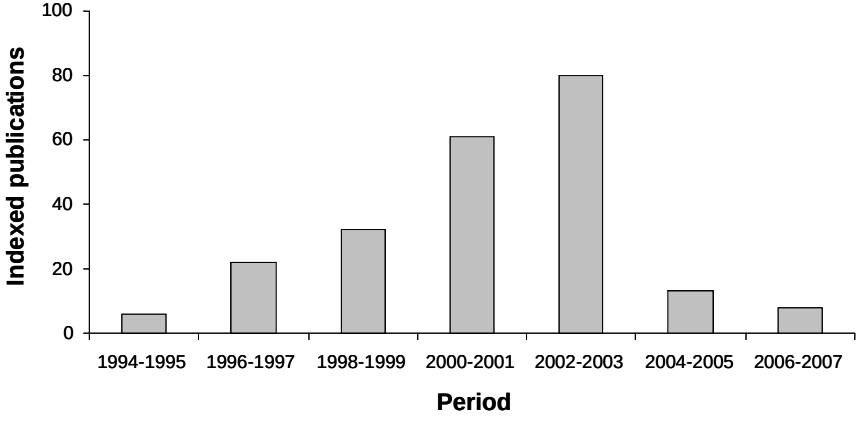

Fig. 1. Number of indexed publications on anti-*Candida* activity around the world for the last fourteen years.

were already studied, but taking into account the existence of around 300,000 plant species around the world much work is yet to be done. In addition, for most of the plants only one part such as the leaves, roots, or stems, or only one type of preparation such as oil, aqueous or ethanolic extracts were studied.

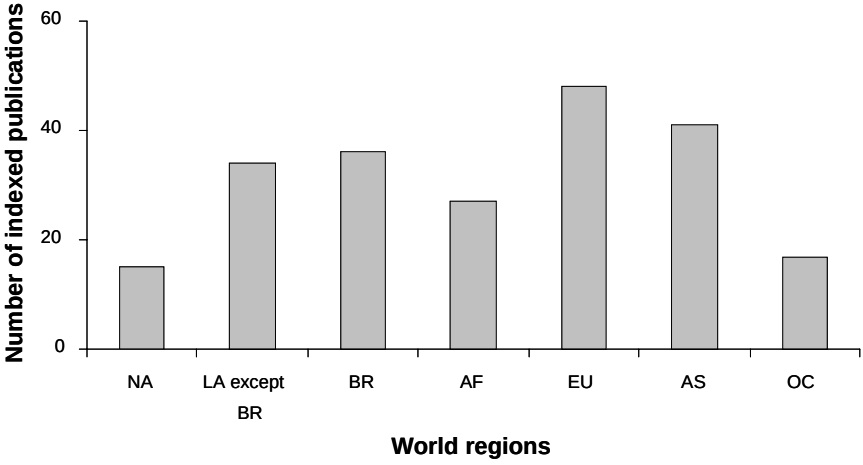

Fig. 2. Publications on anti-*Candida* activity around the world on the last ten years: NA—North America; LA—Latin America; AF—Africa; EU—Europe; AS—Asia; OC—Oceany.

The survey also shows that many of the works focused on the crude extract or oil. The essential oil majority compounds are generally determined and the antimicrobial activity is assigned to a number of small terpenoid and phenolic compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity (Didry et al., 1993; Conner, 1993; Smid et al., 1996; Helander et al., 1998). The antimicrobial activity has been attributed mainly to compounds such as, thymol, geranial, limonene, carvone, carvacrol, menthol and muurolene. However, specific mechanisms of bacterial action of these essential oil constituents remain poorly characterized. The antibacterial properties of these compounds have been associated with their lipophilic character, leading to accumulation in membranes and to subsequent membrane-associated events such as energy depletion (Conner, 1993; Sikkema et al., 1995). Regarding extracts, there is little investigation which exists concerning fractions or active compounds isolated.

Another aspect to be considered are the differences with respect to the techniques applied to investigate the action of the plants compounds, and the great variation found in the chemical composition of the same plant

Table 1

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
ACORACEAE			
Acorus calamus	rh	et	Ahmad and Beg, 2001
AGAVACEAE			
Agave lecheguilla	lv	et	Verástegui et al., 1996
ALANGIACEAE			
Alangium salvifolium	rt	dc + et	Kumar et al., 2006
ANACARDIACEAE			
Schinus molle	lv, fr	tt, sq	Quiroga et al., 2001
Sclerocarya birrea	rt	me	Hamza et al., 2006
ANNONACEAE			
Annona purpurea	wp	et	Lentz et al., 1998
Annona cherimolia	sd	he, me	Navarro-García et al., 2003
Guatteria multivenia	rt	et	Zhang et al., 2002b
Monodora myristica	sd	eo	Cimanga et al., 2002
APIACEAE			
Ammoides pusilla	ap	eo	Laouer et al., 2003
Anethum graveolens	sd	eo	Jirovetz et al., 2003
Coriandrum sativum	lv or fw or bu	ae	Srinivasan et al., 2001
Daucus carota	sd	he	Momin et al., 2002
Hippomarathrum microcarpum	ap	eo	Özer et al., 2007
APOCYANACEAE			
Decalepis hamiltonii	rt	eo	Thangadurai et al., 2002
Holarrhena antidysenterica	bk	et	Ahmad and Beg, 2001
Schizozygia coffaeoides	lv	pe, dc, ea, me	Kariba et al., 2001
Tabernaemontana elegans	rt	aq	Steenkamp et al., 2007
Thevetia lucida	fr, lv, st	et	Lentz et al., 1998
Thevetia nerrifolia	lv	dc + et	Kumar et al., 2006
ARACEAE			
Acorus calamus	rh	et	Ahmad and Beg, 2001
Phylodendron solimoesense	st	me	Jovel et al., 1996
ARALIACEE			
Dendropanax arboreus	fr, lv, st	et	Lentz et al., 1998
ARISTOLOCHIACEAE			
Aristolochia monticola	n.s.	et	Murillo-Alvarez et al., 2001

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
Aristolochia brevipes	n.s.	et	Murillo-Alvarez et al., 2001
ASCLEPIADACEAE			
Cryptolepis sanguinolenta	*rt*	*et*	Silva et al., 1996
Hemidesmus indicus	rt	et	Ahmad and Beg, 2001
Hemidesmus indicus	rt	dc + et	Kumar et al., 2006
ASTERACEAE			
Achillea millefolium	he	me, eo	Candan et al., 2003
Achillea setacea	ap	eo	Unlu et al., 2002
Achillea teretifolia	ap	eo	Unlu et al., 2002
Artemisia asiatica	ap	eof	Kalemba et al., 2002
Artemisia absinthium	ap	eo	Juteau et al., 2003
Artemisia mexicana	lv	me	Navarro et al., 1996
Artemisia herba-alba	ap	et	Khafagi et al., 2000
Baccharis notosergilia	lv	eo	Cobos et al., 2001
Echinops hussonii	ap	et	Khafagi et al., 2000
Felicia erigeroides	rt	et	Salie et al., 1996
Gnaphalium americanum	n.s.	ce	Rojas et al., 2001
Gnaphalium oxyphylum	ap	ce	Rojas et al., 2001
Helichrysum crispum	st	ce	Salie et al., 1996
Hymenoclea monogyra	n.s.	et	Murillo-Alvarez et al., 2001
Hymenoclea sp.	n.s.	et	Murillo-Alvarez et al., 2001
Inula viscosa	lv	bb	Maoz and Neeman, 2000
Osmitopsis asteriscoides	Ap	eo	Viljoen et al., 2003
Ophryosporus peruvianus	lv, st	et	Rojas et al., 2003
Santolina chamaecyparissus	ap	eo	Suresh et al., 1997
Senecio culcitioides	ap	et	Rojas et al., 2003
Senecio graveolens	lv	eo	Pérez et al., 1999
Soroseris hookeriana	ap	mt, sq	Meng et al., 2000
Tagetes lucida	lv	et	Cáceres et al., 1998
BERBERIDACEAE			
Mahonia aquifolium	n.s.	al	Cernakova and Kostalova, 2002
BIGNONIACEAE			
Crescentia alata	fr	ce	Rojas et al., 2001
Tabebuia avellanedae	bk	dc	Portillo et al., 2001

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
BIXACEAE			
Bixa orellana	fr	dc + et	Kumar et al., 2006
BORANGINACEAE			
Alkanna orientalis	ap	he, ea, et	Khafagi et al., 2000
Alkanna strigosa	rt	et	Ali-Shtayeh et al., 1998
Cordia curassavica	rt	qn	Ioset et al., 2000
BRASSICACEAE			
Brassica oleracea	lv	ae	Sisti et al., 2003
Matthiola arabica	ap	et	Khafagi et al., 2000
BURSERACEAE			
Boswellia serrata	rs	dc + et	Kumar et al., 2006
Commiphora mukul	rs	dc + et	Kumar et al., 2006
CAPPARACEAE			
Cleome droserifolia	ap	ea, et	Khafagi et al., 2000
CARYOPHYLACEAE			
Dianthus coryophyllum	lv	et	Erturk, 2006
CASUARINACEAE			
Casuarina equistifolia	lv, bk	et	Ahmad and Beg, 2001
COMBRETACEAE			
Combretum fragrans	rt	me	Fyhrquist et al., 2002
Combretum hereroense	st bk	me	Fyhrquist et al., 2002
Combretum molle	lv	me	Fyhrquist et al., 2002
Combretum molle	rt	me	Steenkamp et al., 2007
Combretum padoides	st bk	me	Fyhrquist et al., 2002
Combretum psidioides	lv	me	Fyhrquist et al., 2002
Combretum zeyheri	rt, st, bk	me	Fyhrquist et al., 2002
Quisqualis indica	st	dc + et	Kumar et al., 2006
Terminalia macroptera	rt	et	Silva et al., 1997
Terminalia alata	bk	me	Taylor et al., 1996
Terminalia alata	rt	et	Silva et al., 1996
Terminalia belerica	fr	et	Ahmad and Beg, 2001
Terminalia chebula	fr	et	Ahmad and Beg, 2001
Terminalia alata	rt	gl	Srivastava et al., 2001
Terminalia kaiserana	rt	me	Fyhrquist et al., 2002
Terminalia sambesiaca	rt, sd	me	Fyhrquist et al., 2002
Terminalia sericea	rt, lv	ae, et, me	Fyhrquist et al., 2002
COMPOSITAE			
Achillea millefolium L.	ap	dc + et	Kumar et al., 2006
Eclipta alba	rt	dc + et	Kumar et al., 2006

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
CRUCIFERAE			
Mathiola arabica	ap	et	Khafagi et al., 2000
Raphanus sativus	sd	dc + et	Kumar et al., 2006
CUCURBITACEAE			
Bryonia syriaca	ap	bu	Mahasneh et al., 1999
Citrullus colocynthis	ap	et	Khafagi et al., 2000
Luffa echinata			
Momordica dioica	fr	dc + et	Kumar et al., 2006
Momordica dioica	rt	dc + et	Kumar et al., 2006
Trichosanthes anguina	fr	dc + et	Kumar et al., 2006
CUNONIACEAE	fr	dc + et	Kumar et al., 2006
Cunonia macrophylla	lv, fw	me	Fogliani et al., 2002
CUPRESSACEAE			
Juniperus oxycedrus	lv	me, eo	Karaman et al., 2003/ Angioni et al., 2003
Juniperus oxycedrus	lv	et	Erturk, 2006
Juniperus phoenicea	lv	eo	Angioni et al., 2003
EBENACEAE			
Diospyros anisandra	lv	np	Ankli et al., 2002
Diospyros cuneata	lv	np	Ankli et al., 2002
EUPHORBIACEAE			
Acalypha guatemalensis	lv	et	Cáceres et al., 1998
Acalypha indica	lv, st, rt	me	Solomon et al., 2005
Croton ruizianus	lv, st	et	Rojas et al., 2003
Chrozophora obliqua	ap	et	Khafagi et al., 2000
Emblica officinalis	fr	et	Ahmad and Beg, 2001
Emblica officinalis	fr	et	Ahmad and Beg, 2001
Mallotus philippinensis	fr	dc + et	Kumar et al., 2006
Spirotachys africana	st	me	Hamza et al., 2006
FABACEAE			
Afzelia quanzensis	bk	aq	Steenkamp et al., 2007
Colutea arborescens	lv	et	Erturk, 2006
Desmodium molliculum	ap	et	Rojas et al., 2003
Lysiloma acapulcensis	bk	he, me	Navarro et al., 2003
Tamarindus indica	lv or fw or bu	ae	Srinivasan et al., 2001
FICOIDACEAE			
Gisekia pharnaceoides	wp	dc + et	Kumar et al., 2006
GENTIANACEAE			
Gentiana macrophylla	rt	n.s.	Chen, 1996

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
GUTTIFERAE			
Mesua ferrea	fl	dc + et	Kumar et al., 2006
HYDROPHYLLACEAE			
Wigandia urens	lv, sts	et	Rojas et al., 2003
HYPERICACEAE			
Hypericum scabrum	ap	ce, ae	Sokmen et al., 1999
Hypericum maculatum	n.s.	eo	Gudzic et al., 2002
JUGLANDACEAE			
Juglans cinerea	bk	et	Omar et al., 2000
Juglans neotropica	bk	me	Lopez et al., 2001
Juglans regia	lv, fr	et	Ali-Shtayeh et al., 1998
LAMIACEAE			
Leucaena glauca	sd	dc + et	Kumar et al., 2006
Calamintha officinalis	ap	eo	Nostro et al., 2002
Lepechinia meyenii	ap	et	Rojas et al., 2003
Mentha piperita	ap	eo	Mimica-Dukic et al., 2003
Origanum syriacum	ap	he, ea	Khafagi et al., 2000
Ocimum sanctum	wp	et	Ahmad and Beg, 2001
Origanum scabrum	ap	eo	Aligiannis et al., 2001
Origanum microphyllum	ap	eo	Aligiannis et al., 2001
Phlomis aurea	ap	et	Khafagi et al., 2000
Plectranthus ornatus	ap	ae	Rijo et al., 2002
Plectranthus amboinicus	lv	eo	Oliveira et al., 2007
Rosmarinus officinalis	ap	he, ea, et	Khafagi et al., 2000
Salvia fruticosa	lv	et	Ali-Shtayeh et al., 1998
Satureja hortensis	ap	me	Sahin et al., 2003
Thymus capitatus	lv	eo	Cosentino et al., 1999
Thymus herba-barona	lv	eo	Cosentino et al., 1999
Thymus fallax	ap	he	Sokmen et al., 1999
Tectona grandis	lv or fw or bu	ae	Srinivasan et al., 2001
Tectona mastichina	fw, lv	eo	Faleiro et al., 2003
Tectona camphoratus	fw, lv	eo	Faleiro et al., 2003
LAURACEAE			
Laurus nobilis	lv	et	Erturk, 2006
LEGUMINOSAE			
Abrus precatorius	sd	dc + et	Kumar et al., 2006
Acacia raddiana	ap	et	Khafagi et al., 2000

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
Albizia lebbeck	lv or fw or bu	ae	Srinivasan et al., 2001
Caesalpinia bonducella	fr	dc + et	Kumar et al., 2006
Caesalpinia pulcherrima	lv	cs	Ragasa et al., 2002
Cassia alata	wp	et	Somchit et al., 2003
Cassia angustifolia	n.s.	ae	Srinivasan et al., 2001
Cassia podocarpa	lv	et	Silva et al., 1996
Erythrina poeppigiana	rt	pd	Sato et al., 2003
Lupinus angustifolius	sd	tt, gl	Woldemichael and Wink, 2002
Ononis spinosa	ap	bu	Mahasneh et al., 1999
Tamarindus indica	lv or fw or bu	ae	Srinivasan et al., 2001
LILIACEAE			
Allium cepa	lv	ae	Srinivasan et al., 2001
Allium sativum	lv, bu	et	Ahmad and Beg, 2001
Allium sativum	bu	ae	Srinivasan et al., 2001
Allium sativum	bu	fs, fd	Lemar et al., 2002
Allium sativum	bu	aq, et, ea, he	Motsei et al., 2003
Allium scorodoprasum	bu	ce, ae	Sokmen et al., 1999
Asphodeline lutea	wp, ap	et	Ali-Shtayeh et al., 1998
Dracaena cinnabari			
LYCOPODIACEAE			
Lycopodium cernuum	ap	et	Zhang et al., 2002c
LYRTHACEAE			
Lawsonia inermis	lv	et	Ahmad and Beg, 2001
MALPIGHIACEAE			
Byrsonima crassifolia	bk	et	Cáceres et al., 1998
MALVACEAE			
Abutilon indicum	fr, rt, lv	dc + et	Kumar et al., 2006
MELIACEAE			
Azadirachta indica	bk	eo	SaiRam et al., 2000
Melia azedarach	fr	et	Carpinella et al., 1999
Melia dubia	lv	eo	Nagalakshmi et al., 2003
Turrea holstii	lv	me	Hamza et al., 2006
MENISPERMACEAE			
Cissampelos pariera	wp	dc + et	Kumar et al., 2006
MIRISTICACEAE			
Iryanthera lancifolia	lv, st, bk	et	Rojas et al., 2003

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
MORACEAE			
Cudrania cochinchinensis	rt	et	Fukai et al., 2003
Dorstenia drakena	lv, st	et	Lentz et al., 1998
Ficus religiosa	lv	et	Ahmad and Beg, 2001
MORINGACEAE			
Moringa pterygosperma	lv or fw or bu	ae	Srinivasan et al., 2001
MYROTHAMNACEAE			
Myrothamnus flabellifolius	n.s.	eo	Viljoen et al., 2002
MYRTACEAE			
Backhousia citriodora	lv	eo	Hayes and Markovic, 2002
Eucalyptus globosus	lv	me	Navarro et al., 1996
Eucalyptus globosus	lv	eo	Cimanga et al., 2002
Eucalyptus sp.	lv	et	Ahmad and Beg, 2001
Eucalyptus tereticornis	lv	eo	Cimanga et al., 2002
Eucalyptus alba	lv	eo	Cimanga et al., 2002
Eucalyptus camaldulensis	lv	eo	Cimanga et al., 2002
Eucalyptus citriodora	lv	eo	Cimanga et al., 2002
Melaleuca alternifolia	lv	eo	Carson and Riley, 1995; Nenoff et al., 1996; Hammer et al., 1998, 1999, 2000; Concha et al., 1998; Cox et al., 2000; D´Auria et al., 2001; Mondello et al., 2003
Mirciria floribundia	bk	me	Jovel et al., 1996
Syzygium aromaticum	bd	et, eo	Ahmad and Beg, 2001
NYMPHACEAE			
Nelumbo nucifera	fw	et	Ahmad and Beg, 2001
OLEACEAE			
Olea europaea	lv	eo	Markin et al., 2003
ONAGRACEAE			
Epilobium angustifolium	rt	et	Jones et al., 2000
Oenothera multicaulis	ap	et	Rojas et al., 2003
PAPAVERACEAE			
Bocconia arborea	lv	me	Navarro et al., 1996
Chelidonium majus	rt	et	Kokoska et al., 2002
PAPILIONACEAE			
Retama raetam	lv, st and yb	et	Ali-Shtayeh et al., 1998

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
PHYTOLACCACEAE			
Petiveria alliacea	lv, st	et	Lentz et al., 1998
PINACEAE			
Abies lasiocarpa	n.s.	fs, pp	Ritch-Krc et al., 1996
Abies webbiana	lv	dc + et	Kumar et al., 2006
Picea engelmannii x Picea glauca	n.s.	fs, pt	Ritch-Krc et al., 1996
Pinus contorta		fs	Ritch-Krc et al., 1996
Pinus brutia	rt, st	me, he, ce, pe, ea bu	Kizil et al., 2002
Pinus gerardiana	nt	dc + et	Kumar et al., 2006
PIPERACEAE			
Piper aduncum	fr, Lv, st	et	Lentz et al., 1998
Piper lanceaefolium	lv	me	Lopez et al., 2001
Piper capense	bk	me	Steenkamp et al., 2007
PLANTAGINACEAE			
Plantago lancelata	sd	dc + et	Kumar et al., 2006
Plantago major	lv	me	Navarro et al., 1996
PLUMBAGINACEAE			
Plumbago zeylanica	rt	et	Ahmad and Beg, 2001
Plumbago zeylanica	rt	dc + et	Kumar et al., 2006
POACEAE			
Cymbopogon citratus	lv	eo	Cimanga et al., 2002
POLYGALACEAE			
Polygala myrtifolia	lv	aq, et, ea, he	Motsei et al., 2003
POLYGONACEAE			
Rheum emodi	rhs	me	Agarwal et al., 2000
PRIMULACEAE			
Anagallis arvensis	wp	et	Ali-Shtayeh et al., 1998
Cyclamen persicum	ap	bu	Mahasneh et al., 1999
PTERIDACEAE			
Pityrogramma calomelanos	lv	et	Lentz et al., 1998
PUNICACEAE			
Punica granatum	bk	et	Ahmad and Beg, 2001
PYROLACEAE			
Chimaphila umbellata	wp	et	Jones et al., 2000
RANUNCULACEAE			
Clematis hirsuta	lv	fs	Cos et al., 2002
Coptis trifolia	wp	et	Jones et al., 2000

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
Nigella sativa	sd	ce	Sokmen et al., 1999; Khan et al., 2003
RHAMNACEAE			
Zizyphus jujuba	lv, bk	et	Ahmad and Beg, 2001
ROSACEAE			
Rubus apelatus	st, lv, fr	me	Hamill et al., 2003
Sarcopoterium spinosum	rt, fr, sds	et	Ali-Shtayeh et al., 1998
RUTACEAE			
Aegle marmelos	fr	dc + et	Kumar et al., 2006
Citrus sinensis	bk	et	Ahmad and Beg, 2001
Clausena anisata	bk	me	Hamza et al., 2006
Murraya exotica	lv	dc + et	Kumar et al., 2006
Ruta chalepensis	lv, rt, wp	et	Ali-Shtayeh et al., 1998
Zanthoxylum lepieurii	lv, rt, st, bks	aq, et	Ngane et al., 2000
Zanthoxylum xanthoxyloides	st, bks	aq, et	Ngane et al., 2000
SAPINDACEAE			
Sapindus sp.	frs		
SAXIFRAGACEAE			
Bergenia crassifolia	ap, rh	et	Kokoska et al., 2002
SCROPHULARIACEAE			
Verbascum sinaiticum	ap	he, ea, et	Khafagi et al., 2000
Verbascum spp	n.s.	fs	Dulger et al., 2002
SMILACACEAE			
Smilax lundellii	rh	et	Cáceres et al., 1998
SOLANACEAE			
Capsicum spp	ts	ae	Cichewicz et al., 1996
Cestrum auriculatum	lv	et	Rojas et al., 2003
Lycianthes synanthera	ap	me	Nino et al., 2006
Lycium barbarum	br	dc + et	Kumar et al., 2006
Pentagonia gigantifolia	rt	et	Li et al., 2003
Solanum aculeastrum	fr	me	Steenkamp et al., 2007
Solanum indicum	br	dc + et	Kumar et al., 2006
Solanum mammosum	fw	me	Jovel et al., 1996
STERCULIACEAE			
Helectres isora	fr	dc + et	Kumar et al., 2006
Guazuma ulmifolia	bk, lv	et	Lentz et al., 1998
SYMPLOCACEAE			
Symplocos racemosa	st, bk	dc + et	Kumar et al., 2006

Contd.

Table 1 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
TAXACEAE			
Taxus baccata	lv	dc + et	Kumar et al., 2006
URTICACEAE			
Parietaria alsinifolia	ap	et	Khafagi et al., 2000
Parietaria diffusa	ap	et	Ali-Shtayeh et al., 1998
VERBENACEAE			
Lantana xenica	ap	eo	Juliani et al., 2002
Vitex nigundo	lv	dc + et	Kumar et al., 2006
VIOLACEAE			
Leonia glycicarpa	bk	me	Jovel et al., 1996
VITACEAE			
Cyphostemma flaviflorum	rt	me	Lin et al., 1999
Cyphostemma lanigerum	rt	me	Lin et al., 1999
Cyphostemma sp.	lv, rt	me	Lin et al., 1999
Cyphostemma natalitium,	lv, st, rt	me	Lin et al., 1999
Rhoicissus digitata	rt	me	Lin et al., 1999
Rhoicissus rhomboidea	st, rt	me	Lin et al., 1999
Rhoicissus tomentosa	lv, st	me	Lin et al., 1999
Rhoicissus tridentata	rt	me	Lin et al., 1999
ZINGIBERACEAE			
Aframomum danielli	fr	eo	Martins et al., 2001
Zingiber officinale	rhs	eo	Martins et al., 2001
Zingiber officinale	lv or fw or bu	ae	Srinivasan et al., 2001
ZYGOPHYLACEAE			
Fagonia arabica			
Fagonia mollis	ap	he, ea	Khafagi et al., 2000
Zygophyllum coccineum	ap	et	Khafagi et al., 2000
Perganum harmala	sd	ce, ae	Sokmen et al., 1999/ Khan et al., 2003

[a]lv, leaves; wp, whole plant; sd, seed; fr, fruit; st, stem; bk, bark; br, berry; rh, rhizome; rs, resin; rt, root; ap, aerial parts; he, herbal parts; fw, flower; bu, bulb; bd, bud; yb, young branches; ts, tissues; nt, nut.

[b]**Extracts**: fs, fresh; aq, aqueous; et, ethanolic; me, methanolic; ce, chloroform; ac, acetone; bu, buthanol; he, hexane; fd, freeze dried; ea, ethyl acetate; dc, dichloromethane; pe, petroleum ether; np, non polar; pl, polar. **Oil**: eo, essential oil; eof, essential oil fraction; pt, pitch preparation. **Derivatives**: mt, monoterpene; sq, sesquiterpene; tt, triterpenes; pd, phytochemical; cs, cassave; qn, quinines; gl, glycosides. **Others**: bb, borate buffer; al, alkaloid.

n.s., not specified.

preparations, which can yield results that are difficult to reconcile (Sivropoulou et al., 1995). One of the most common assays employed is the disk diffusion test, and although the data obtained from this method are qualitative and poor indicators of the relative antimicrobial activity, since the size of the cleared zone is dependent on the solubility and rate of diffusion of the sample in the aqueous medium, it is a practical way of screening large numbers of materials (Morris et al., 1979). Minimal Inhibitory Concentration (MIC) quantitative test is also used frequently, but are not necessarily comparable with the first method.

Finally, the controls utilized to evaluate the efficiency of plants compounds are standard antibiotics as indicated for each microorganism. However, there is no an agreement on what level is acceptable for plant compounds when compared with standards, so some authors consider only results similar to antibiotics, while others consider even higher levels.

Anti-Candida activity from traditional medicinal plants in Brazil

Many plants from Brazilian biomes, such as Cerrado (savannah), the Atlantic and Amazon rain forests, have been used as natural medicines by local populations in the treatment of several tropical diseases, including schistosomasis, leishmaniasis, malaria, fungal and bacterial infections (Alves et al., 2000). Moreover, many exotics plants were introduced in Brazil since its colonization, and have been incorporated into folk medicine.

The investigation of natural products active against *Candida* spp. in Brazil also increased significantly during recent years as can be seen from Figure. 2, which shows the number of indexed and non indexed papers (ISI) in the last 10 years. As in other world regions, scientific investigations are guided from a ethnopharmacological survey. However, despite the rich flora, only data from about 44 plant species from 20 families with positive activity are available, including both native and exotic species, partly due to the restricted dissemination of research results, disclosed in local/regional scientific meetings and publications.

Regarding publications of anti-*Candida* activity, the number of works carried out in Brazil (indexed and non) are similar to those produced in the rest of Latin America and Asia (Figure 2).

In Brazil, most of the research focuses on isolated tests with one or a few species generally based on ethnopharmacological information, different from research which focuses on the flora from a defined region, where several botanic families are studied. A wide study should be more effective if the investigation ranges the pharmacological potential of several species from one determinate genus guided by folk medicinal use, such as the study carried out by Silva and Cechinel (2002) on plants of the genus *Bauhinia*

spp. The authors studied the chemical composition and pharmacological potential of 16 extracts against Gram-positive and Gram-negative microorganisms. Therefore, no activity was founded against *C. albicans*.

Due to the great biodiversity present in the different biomes from Brazil, there is a growing demand for natural products by national and international pharmaceutical industries that impulses the scientific investigation and the search for natural drugs. This sequence of events resulted in a 'sui generis' legislation regarding biodiversity and traditional knowledge now put into effect.

Methods—A model study in Brazil

In a recent study carried out at the Pluridisciplinar Research Center for Chemistry, Biology and Agriculture—CPQBA/State University from Campinas—São Paulo—Brazil, 45 native and exotic medicinal plants popularly used in Brazil were selected to be screened against *C. albicans* ATCC 10231, based on their traditional uses (Duarte et al., 2005).

A list of the plants studied, including the botanical name, voucher specimen and data related to traditional use and plant parts used, are listed in Table 3. The plants were grown in the experimental field of the CPQBA/UNICAMP and were collected from November/2001 to November/2002. Voucher specimens were deposited at the State University of Campinas Herbarium (UEC) or at the herbarium at the CPQBA. The specimens referred to as *Mentha* sp. are currently under identification at the Kew Gardens (London, UK).

The essential oils were obtained from 40 g of fresh plant parts by water-distillation using a Clevenger-type system for 3 h. The aqueous phase was extracted three times with 50 mL dichloromethane. The pooled organic phases were dried with sodium sulfate, filtered and the solvent evaporated until dry. Dried samples were stored at -25ºC in sealed glass vials.

Extracts were obtained from dried plant material (10 g) macerated with 100 mL of 70% ethanol and submitted to shaking at 200 rpm at room temperature for 3 h. Subsequently, the extracts were filtered and the plants residues were re-extracted with fresh 70% ethanol. The pooled filtrates were concentrated under vacuum and stored at 4ºC until further use.

Candida albicans CBMAI 0475 (ATCC 10231) was obtained from the CBMAI (Brazilian Collection of Environmental and Industrial Microorganisms, CPQBA/UNICAMP, Brazil). The yeast was grown overnight at 36ºC in Sabouraud Dextrose Agar (Merck) plates, and inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometrical reading at 580 nm. Cell suspensions were finally diluted to 10^4 UFC/mL

for being used in the activity assays.

MIC tests were carried out according to Eloff (1998), using a tissue culture testplate (96 wells). The stock solutions of the extracts and oils were diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 2-0.03 mg/mL were obtained. Nistatin (Merck) was used as the reference antimycotic control in the range of 0.005-0.060 mg/mL. The yeast inoculum was added to all wells and the plates were incubated at 36ºC for 48 h. Antimicrobial activity was detected by adding 20 µL of 0.5% TTC (triphenyl tetrazolium chloride, Merck) aqueous solution. MIC was defined as the lowest concentration of oil and extract that inhibited visible growth, as indicated by the TCC staining (dead *C. albicans* cells are not stained by TTC).

The identification of volatile constituents was performed using a Hewlett-Packard 5890 Series II gas chromatograph, equipped with a HP-5971 mass selective detector and capillary column HP-5 (25 m × 0.2 mm × 0.33 µm diam.). GC and GC-MS were done using *split/splitless* injection, with injector set at 220ºC, column set at 60ºC, with heating ramp of 3ºC.min^{-1} and final temperature 240ºC for 7 min, and the FID detector set at 250ºC. Helium was used as carrier gas at 1 mL.min^{-1}. The GC-MS electron ionization system was set at 70 eV. A sample of the essential oil was solubilized in ethyl acetate for the analyses. Retention indices (RI) were determined by co-injection of hydrocarbon standards. The oil components were identified by comparison with data from literature (Adams, 2001), the profiles from the Wiley 138 and Nist 98 libraries, and by co-injection of authentic standards, when available.

Oil and extract yields

Oil and extracts yields of the plant parts indicated in Table 4, expressed in relation to dry weight plant material, are presented in Table 5. Most plants had oil yield below 1% (w/w), though larger quantities were obtained from *Mentha* sp. (3.5% w/w).

Anti-Candida activity

MIC results of the oils and extracts obtained from the plants tested are shown in Table 5. In general, the controls utilized to evaluate the efficiency of plants compounds are standard antibiotics as indicated for each microorganism. However, there is not an agreement on what level is acceptable for a plant when compared with standards, so some authors consider only results similar to antibiotics, while others consider even higher values. Aligianis et al. (2001) proposed a classification for plant materials, based at MIC results as: strong inhibitors—MIC until 0.5 mg/

mL; moderate inhibitors—MIC between 0.6 and 1.5 mg/mL and weak inhibitors—MIC above 1.6 mg/mL. Thus, in our work we used 2.0 mg/mL as the highest concentration and only the oils or extracts presenting MIC below 2.0 mg/mL were considered as potential antimicrobial. Therefore, Table 2 indicates a strong activity against *C. albicans* for oils from *A. millefolium*, *M. glomerata* and *Stachys bizantina* (MIC = 0.25 mg/mL). *A. triphyla*, *A. nobilis*, *C. martinii*, *C. articulatus*, *C. rotundus L. alba*, *M. arvensis*, *M. piperita*, *M.* sp. and *S. chilensis* presented moderate activity, while in the range stipulated as weak activity was showm by *B. dracunculifolia*, *O. vulgare*, *Piper regnelli* and *T. vulgaris*. Regarding the standard used in the tests, the MIC for nistatin was 0.05 mg/mL. All remaining plants and extracts investigated presented MIC above 2.0 mg/mL.

Table 2

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
AMARANTACEAE			
Alternanthera maritima	ap	ae, et	Salvador et al., 2002a
Blutaparon portulacoides	ap, rt	gl	Salvador et al., 2002b
ANACARDIACEAE			
Anacardium ocidentale	lv	ns	Queiroz et al., 2000
Myracrodruon urundeuva	lv	ha	Paiva et al., 2002
ANONACEAE			
Annona crassiflora	lv	et	Ferreira et al., 1998; Silva et al., 2001
Annona coriaceae	lv	et	Ferreira et al., 1998; Silva et al., 2001
ASTERACEAE			
Artemisia annua	ap	lseq	Foglio et al., 2002
Calea serrata	lv	eo	Flach et al., 2000
Conyza bonariensis	n.s.	eo	Lima et al., 1994a
BORANGINACEAE			
Cordia nodosa	rt	ea, me	Costa et al., 2002a
BURSERACEAE			
Bursera simaruba	fr	eo	Savi et al., 1996
Protium heptaphyllum	lv	eo	Lemos et al., 2002
Protium tenuifolium	fr, re	eo	Lemos et al., 2002
CHRYSOBALANACEAE			
Licania tomentosa	lv, fr	he, me	Castilho et al., 2002

Contd.

Table 2 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
EUPHORBIACEAE			
Croton spp.	n.s.	eo	Cunha et al., 1994
Croton nepetaefolius	lv	eo	Morais et al., 2002
Phyllanthus sellowianus	ap	al	Cechinel Filho et al., 1994
Sebastiania schottiana	lv	xa	Lima et al., 1994b
FABACEAE			
Bowdichia virgilioides	n.s.	eo	Lima et al., 1994a
Pitecellobium avaremotemo	lv	ns	Queiroz et al., 2000
FLACOURTIACEAE			
Casearia sylvestris	lv	ae	Sato et al., 1996
GUTTIFERAE			
Kielmeyera coriacea	ap	pl	Cortez & Cortez, 1998
LAMIACEAE			
Hyptis sp.	n.s.	eo	Lima et al., 1994a
Mentha piperita	ap	eo	Santoratto et al., 2002
Mentha sp.	lv	eo	Santos et al., 2000a
Ocimum gratissimum	n.s.	eo	Lima et al., 1994a
Ocimum gratissimum	ap	eo	Nakamura et al., 1996
Plectranthus amboinicus	lv	ns	Queiroz et al., 2000
MALPIGHIACEAE			
Mascagnia rigida	lv, rt	ch, ea, me	Costa et al., 2002b
MONIMIACEAE			
Peumus boldus	n.s.	eo	Lima et al., 1994a
MYRTACEAE			
Psidium spp.	n.s.	eo	Cunha et al., 1994
PIPERACEAE			
Piper cernuum	lv	eo	Constantin et al., 2001
Piper marginatum	lv	eo	Lima et al., 1996
Piper regnelli	lv	eo	Constantin et al., 2001
POACEAE			
Cymbopogon citratus	lv	eo	Santos et al., 2000b
PUNICACEAE			
Punica granatum	fr	ae	Lima et al., 2002
Punica granatum	fr	ns	Queiroz et al., 2000
RUBIACEAE			
Borreria cupularia	n.s.	eo	Lima et al., 1994a
Rubus rosaefolius	lv	ae, et	Mauro et al., 2000

Contd.

Table 2 continued

Family and Botanical name	*Plant part*[a]	*Extract, Essential oil, Derived*[b]	*References*
RUTACEAE			
Pilocarpus trachyllophus	n.s.	eo	Melo et al., 1994
SOLANACEAE			
Solanum grandiflorum	fr	et	Ferreira et al., 1998; Silva et al., 2001
Solanum lycocarpum	fr	et	Ferreira et al., 1998; Silva et al., 2001
VERBENACEAE			
Aloysia tryphila	ap	eo	Sartoratto et al., 2002
Lippia sidoides	ap	eo, me, et, ae	Nunes et al., 1998

[a]lv, leaves; fr, fruit; rt, root; ap, aerial parts.

[b]**Extracts**: aq, aqueous; ha, hydroalcoholic; me, methanolic; ch, ciclohexano; ea, ethyl acetate; pl, polar.

Oil: eo, essential oil. **Derivatives**: lsq, lactones sesquiterpenes; gl, glycosides; xa, xantoxilin. **Others**: re, resin; al, alkaloids.

n.s., not specified.

Chemical characterization of oil constituents

Oils that presented anti-*Candida* activity at levels below 2.0 mg/mL were subjected to GC and GC-MS analyses (Table 3). The majority of the oil constituents were identified using the data sources available, except in the case of *Anthemis nobilis*, where only 40.30% of the oil components could be identified. Among the identified compounds, some were previously reported to have antimicrobial activity, including 1,8-cineole, limonene and linalool (Mazzanti et al., 1998), geranial (Araujo et al., 2003), germacrene-D (Ngassapa et al., 2003), and menthol (Iscan et al., 2002). The oil components with molecular masses ranging from 202 to 222 g/mol, which consisted of sesquiterpenes, could be not identified.

In conclusion, the results of the present study indicate that essential oils obtained from 13 of the 41 plants commonly used in Brazilian folk medicine had anti-*Candida* activity. Essential oil of three plants, namely *A. millefolium, M. glomerata* and *Stachys bizantina* presenting strong activity, at levels 0.25 mg/mL will be further characterized. This study corroborates the importance of ethnopharmacology survey data in the selection of plants for bioactivity screening. The results obtained represent an expressive contribution to the characterization of the anti-*Candida* activity of essential oils and plant extracts for traditional medicinal plants of the Brazilian flora, as previous studies have not focused into this type of assays.

Table 3

Botanical name	*Family*	*Voucher*	*Origin*[a]	*Plant part*[b]	*Traditional use*[c,d]
Achillea millefolium L.	Asteraceae	UEC 127.114	E	lv	antinflammatory, healing
Achyrocline satureoides (DC.) Lam.	Asteraceae	UEC 127.116	N	lv	antinflammatory, analgenic
Allium schoenoprasum L.	Liliaceae	UEC 121.397	E	lv, rt	digestive, antibiotic, analgenic
Aloysia gratissima (Gill and Hook)	Verbenaceae	UEC 121.393	N	lv	spice, digestive, sedative
Aloysia triphylla (L'hér.) Britten.	Verbenaceae	UEC 121.412	E	lv	spice, digestive, sedative
Anthemis nobilis L.	Asteraceae	UEC 121.411	E	lv	antispasmodic, aromatic, digestive
Artemisia annua L.	Asteraceae	CPQBA 1246	E	lv	disinfectant, antimalaric
Baccharis dracunculifolia Dc.	Asteraceae	CPQBA 622	N	lv	antibiotic
Baccharis. trimera (Less.) Dc.	Asteraceae	CPQBA 1	N	lv	digestive, antihelminthic
Cordia curassavica (Jacq.) Roem.	Boraginaceae	UEC112744	N	lv	antinflammatory
Cymbopogon martinii Motia.	Poaceae	UEC 127.115	E	lv	antiseptic, repellent
Cymbopogon. winterianus L.	Poaceae	UEC 121.414	E	lv	antiseptic, repellent
Cyperus articulatus L.	Cyperaceae	UEC 121.396	N	rt	antibiotic, antinflammatory
Cyperus rotundus L.	Cyperaceae	CPQBA 1252	N	rt	antibiotic, antinflammatory
Hydrocotyle asiatica L.	Apiaceae	UEC 127.111	E	lv	antinflammatory, healing
Lippia alba (Mill) N.E. Br.	Verbenaceae	UEC121413	N	lv	soothing, analgesic
Mentha arvensis var. *piperita* L.	Lamiaceae	CPQBA 8	E	lv	antidispeptic, antivomitive

Contd.

Table 3 continued

Botanical name	*Family*	*Voucher*	*Origin*[a]	*Plant part*[b]	*Traditional use*[c,d]
Mentha piperita L.	Lamiaceae	UEC 127.110	E	lv	antiseptic, vermifug
Mentha pulegium L.	Lamiaceae	UEC 121.402	E	lv	antiseptic, anticough
Mentha sp.	Lamiaceae	CPQBA 1253	E	lv	antiseptic, vermifug
Mentha spicata L.	Lamiaceae	CPQBA 9	E	lv	antispasmodic, diuretic
Mikania glomerata Sprengel	Asteraceae	UEC 102047	N	lv	expectorant, anticold
Mikania laevigata Sch. Bip. ex Baker	Asteraceae	UEC 102044	N	lv	expectorant, anticold
Ocimum basilicum L.	Lamiaceae	UEC 121.408	E	lv	digestive, vermifug
Ocimum gratissimum L.	Lamiaceae	UEC 121.407	N	lv	anticold, diuretic
Ocimum selloii Benth.	Lamiaceae	UEC 121.406	N	lv	gastritis, expectorant
Origanum applii (Domin) Boros	Lamiaceae	UEC 121.410	E	lv	analgesic, expectorant
Origanum vulgare subsp. *virens* L.	Lamiaceae	UEC 121.409	E	lv	analgesic, expectorant
Piper aduncum L.	Piperaceae	UEC 127.118	N	lv	tonic, antispasmodic
Piper marginatum Jacq.	Piperaceae	UEC 121.395	N	lv	tonic, antispasmodic
Piper regnellii (Miq.) C.DC.	Piperaceae	UEC 127.120	N	lv	tonic, antispasmodic
Plectranthus barbatus Benth.	Lamiaceae	UEC 121.403	N	lv	gastritis, dispepsy
Potomorphe umbellta (L.) Miquel	Piperaceae	UEC 127.123	N	lv	diuretic, antiepleptic, antipyrectic
Solidago chilensis Meyen	Asteraceae	UEC 121.391	N	lv	adstringent
Spilanthes acmella L.	Asteraceae	UEC 127.272	N	lv	analgesic, dispepsy

Contd.

Table 3 continued

Botanical name	*Family*	*Voucher*	*Origin*[a]	*Plant part*[b]	*Traditional use*[c,d]
Stachys byzantina C. Koch.	Lamiaceae	UEC 121.404	E	lv	antinflammatory
Stachytarphetta cayenensis (L.C.)	Verbenaceae	UEC 121.394	N	lv	tonic, diuretic, stimulant
Stevia rebaudiana (Bert)	Compositeae	CPQBA 72	E	lv	swetener, tonic
Thymus vulgaris L.	Lamiaceae	UEC 121.405	E	lv	antiseptic, antispasmodic
Tropaeolum majus L.	Tropeolaceae	UEC 121.416	E	lv	antiseptic, expectorant
Vernonia condensata Baker	Asteraceae	UEC 121.399	N	lv	gastritis, dispepsy
Vetiveria zizanoides Stapf.	Poaceae	UEC 121.415	E	lv, rt	aromatic
Viola odorata L.	Violaceae	UEC127108	E	lv	emetic, expectorant

[a]N = native to Brazil; E = exotic; [b]lv, leaves; rt, roots. [c]Data from Lorenzi and Matos (2002) and [d]Lust (1983).

Table 4

Plant species[a]	*Yield (% w/w)*[b]		*MIC (mg.mL^{-1})*[b]	
	Essential oil	*Ethanolic extract*	*Essential oil*	*Ethanolic extract*
Achillea millefolium	0.19	61.60	0.25	*
Achyrocline satureoides	0.08	32.70	*	*
Allium schoenoprazum—rt	0.07	26.30	*	*
Allium schoenoprazum—lv	0.09	45.90	*	*
Aloysia gratissina	0.50	20.80	*	*
Aloysia triphyla	0.22	27.00	0.8	*
Anthemis nobilis	0.15	42.90	1.0	*
Artemisia annua	0.35	34.50	*	*
Baccharis dracunculifolia	0.35	46.40	2.0	*
Baccharis trimera	0.07	51.71	*	*
Cordia curassavica	0.10	15.80	*	*
Cymbopogon martinii	0.33	20.90	0.6	*

Contd.

Table 4 continued

Plant species[a]	*Yield (% w/w)*[b]		*MIC (mg.mL^{-1})*[b]	
	Essential oil	*Ethanolic extract*	*Essential oil*	*Ethanolic extract*
Cymbopogon winterianus	0.70	48.50	1.6	*
Cyperus articulatus	0.15	17.33	0.6	*
Cyperus rotundus	0.10	32.30	0.6	*
Hidrocotyle asiatica	0.03	36.30	-	*
Lipia alba	0.26	29.60	1.1	*
Mentha arvensis	0.80	26.80	0.6	*
Mentha piperita	0.42	32,00	0.74	*
Mentha pullegium	0.21	43.60	*	*
Mentha sp.	3.50	n.d.	1.0	-
Mentha spicata	0.32	43.82	*	*
Mikania glomerata	0.05	17.97	0.25	*
Mikania laevigata	0.02	30.00	*	*
Ocimum basilicum	0.10	33.00	*	*
Ocimum gratissimum	0.74	24.00	*	*
Ocimum selloii	0.18	17.33	*	*
Origanum applii	0.20	42.10	*	*
Origanum vulgare	0.13	35.02	2.0	*
Piper aduncum	0.19	32.63	*	*
Piper marginatum	0.30	27.30	*	*
Piper sp.	0.12	27.45	2.0	*
Plectranthus barbatus	0.06	28.60	*	*
Potomorphe umbellata	0.13	30.20	*	*
Solidago chilensis	0.15	35.70	1.5	*
Spilanthes acmella	n.d.	47.70	*	*
Stachys bizantina	0.03	30.02	0.25	-
Stevia rebaudiana	n.d.	47.70	n.d.	*
Thymus vulgaris	0.56	27.00	2.0	*
Trapaeolum majus	0.08	46.80	*	*
Vernonia condensata	0.24	44.22	*	*
Vetiveria zizanoides—lv	0.67	5.77	*	*
Vetiveria zizanoides—rt	0.15	7.54	*	*
Viola odorata	0.06	32.55	*	*

[a]lv, leaves; rt, root; [b]n.d. = not done; * = MIC > 2.0.

Table 5

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
α-pinene	931								0.17					0.86
camphene	945													0.32
sabinene	971													1.14
β-pinene	974					0.39			0.38					0.67
octen-4-ol	976		0.46					0.80						
6-methyl-5-hepten-2-one	984	3.63												
β-myrcene	990	0.74		0.12					0.32			0.42		1.18
3-octanol	998										10.09			
dl-limonene	1026	18.75			1.61				1.59	0.47		0.13		0.48
1.8-cineol	1028		14.04			0.60		2.34				0.13		
cis-β-ocimene	1034			0.23								0.61	1.39	
Trans-β-ocimene	1044	0.82		0.98								0.39		0.92
isopentil butyrate	1068										0.67			
linalool	1101							76.30			51.03	13.59		
dehydro sabine ketone	1117		0.61											
α-camphonelal	1123					0.83								

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
nopinone	1133					2.84								
trans-pinocarveol	1135					17.44	0.63							
trans-verbenol	1141					2.10								
isopulegol	1143				0.99				0.61					
sabine ketone	1153					0.95								
p-mentone	1153								12.00					
citronelal	1154				36.24									
pinocarvone	1160					3.68								
MW 154	1162								4.72					
p-mentha-1.5-dien-8-ol	1164					7.06								
terpin-4-ol	1174		1.38			2.13					8.00			0.90
menthol	1179								69.77					
p-cimen-8-ol	1185					4.44								
$\tilde{\alpha}$-terpineol	1187					3.37		0.49			1.31	4.14		
myrtenal	1193					8.16								
myrtenol	1194					4.61	0.69							

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
verbenone	1209					19.57								
trans-carveol	1217					2.45								
nerol	1226	6.52										0.78		
citronelol	1230				18.43			0.81						
carvone	1240					1.40					23.42			
neral	1240							0.62						
piperitenone	1252								1.39					
trans-geraniol	1254	8.29	6.71	63.46	11.63									
linalil acetate	1262											67.21		
geranial	1269	21.77			0.23			0.83						
isopulegol acetate	1274								0.26					
isoborneol acetate	1284													11.27
menthol acetate	1294								6.98					
δ-elemene	1334									2.24				3.25
citronelil acetate	1352				2.51									
eugenol	1355				1.04									

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
neril acetate	1364											1.15		
ciclosativene	1366						1.79							
α̃-copaene	1370									0.88				
geranil acetate	1382	3.15	1.52	28.83	1.05			0.19				2.46		
β̃-bourbonene	1385										0.18			0.56
β̃-elemene	1387				0.91			0.36		0.79			6.83	
ciperene	1393						3.05							
trans-cariophylene	1414	4.94		2.15				1.91	0.42	14.53	2.31	1.78		
MW 202	1415													3.91
α̃-humulene	1447							0.26		1.87				0.86
α̃-patchoulene	1452						1.34							
trañs-β̃-farnesene	1455		2.84									0.29		
γ̃-muurolene	1477		8.26		1.80				0.47			0.80	65.00	10.05
MW 204	1477						0.52	2.97						
germacrene D	1479									41.45	0.44			
<AR> curcumene	1481	1.43												

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
MW 204	1487						0.80							
biciclogermacrene	1492	2.63	0.31							9.81				
β̃-bisabolene	1509		4.18											
7-EPĨ-α̃-selinene	1515						0.64							
metĩl-α̃-ionone	1518													1.82
cis-calamenene	1520													0.84
δ̃-cadinene	1520				1.66					1.42				
MW 220	1521						2.07							
elemol	1546				7.15					1.97		0.69		0.81
MW 220	1550													1.72
germacrene B	1552							1.91		4.14				
MW 220	1552						0.56							
MW 204	1562							0.53						
trans-nerolidol	1563													0.79
germacrene D-4-ol	1569				1.30			0.19						
spatulenol	1572	2.61								2.97				0.73

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
MW 222	1575												2.12	
cariofilene oxide	1576						3.70			3.48				
veridiflorol	1589											2.43		
1-hexadecene	1592													1.97
MW 204	1626												2.88	
γ̃-eudesmol	1626				1.12									
MW 220	1627													3.26
3-iso-thujopsanone	1636						2.81							
EPĨ-α̃-muurolol	1640				2.58					2.06				
β̃-eudesmol	1644				0.77									
α̃-eudesmol	1649											0.23		
α̃-cadinol	1651				4.77					3.03				1.99
MW = 220	1666													3.09
valeranone	1668												15.42	
MW 220	1675													3.57
MW 218	1676						13.25							

Contd.

Table 5 continued

Compounds[a]	*RI*[b]	*AT*[c]	*AN*	*CM*	*CW*	*CA*	*CR*	*LA*	*MA*	*MG*	*MP*	*Msp*	*SB*	*SC*
MW 218	1687						15.00							
MW 218	1692						21.26							
MW 220	1705													1.99
MW 220	1715													2.69
MW 220	1718													1.32
(E.E) farnesol	1720			1.57										
4-nonil-phenol	1724													2.38
MW 220	1740													3.06
MW 220	1753													2.50
MW 220	1761													1.65
MW 262	1776						5.35							
1-octadecene	1792													2.46
Total		75.28	40.30	97.34	95.77	82.00	77.46	90.51	99.08	91.12	97.45	97.23	93.64	75.01

[a]MW = molecular weight. [b]RI = retention index. [c]Results expressed as % of area.

Subsequently, bioguided fractionation will be conducted to the potential plants for identification of the active compounds. Evaluations of the oils against other important human pathogens are also being conducted.

Conclusion and future prospects

The aim of this survey was to collect the main research on anti-*Candida* activity from medicinal plants in Brazil as well as around the world in the last 10 years, and to be one more source for studies of potential genera or species.

From the consulted literature many families or genera were found to be active to *Candida albicans* strains. These families and genera should be used as another important guide to find different species from the same genera with potential activity once the similarity of chemistry taxonomy is known in chemical compounds among different species growing from several biomes.

Regarding the methodology employed to evaluate the antimicrobial activity, there is an urgency to standardize the method and thus only one technique should be used by different researchers. It is also necessary to establish the acceptable levels of inhibition for plant compounds related to standard antibiotics.

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6 Bridging the Gap: Using Natural Products in Drug Discovery Research in Academia and Industry

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INTRODUCTION

Where do drugs come from?

Historically, natural products were the origin of all medicines. Indeed, an analysis of the chemical 'ancestry' "of medicinal compounds indicated that 80% originated from natural materials (Sneader, 1996; Figure 1). However,

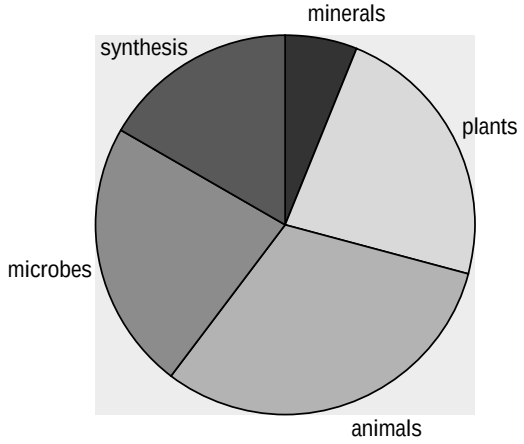

Fig. 1. Origin of the 244 drug prototypes used up to 1995. *Source:* Sneader (1996).

in the 20th century, synthetic chemistry and biotechnology offered alternatives to natural products. For technological convenience, the efforts of drug discovery scientists have tended to be directed at synthetic rather than natural products; however, there is a shortage of successful new chemical entities being introduced to medical practice (Drews, 2000; Baker and Gill, 2005; Cuatrecasas, 2005). This chapter will attempt to show how natural products can still be a useful way to bridge the gap in drug discovery. Studies on natural products can also be a means to link research in academia with that in pharmaceutical companies.

Ethnopharmacology

Broadly speaking, natural products can be used in drug discovery in two main ways: in focused studies directed by information from traditional uses of natural products, and in random screening programmes.

It is often claimed that using ethnopharmacological information will greatly increase the chances of discovering new drugs. However, it is not clear from published information whether this assertion is valid. Two large-scale studies provide some pointers to success rates with natural product screening. The NCI anti-cancer screening programme published that screening of random samples gave a hit rate of 10.4%, while plants collected on the basis of some ethnopharmacological information gave a hit rate of 19.9%. However, plants that were known to be poisonous had a much higher initial hit rate - 50%. The second example comes from the Central Drug Research Institute in Lucknow, India. On a wide range of assays, randomly collected plants had a hit rate of 18.9%, whereas plants with associated ethnopharmacological uses had a hit rate of 18.3%.

More recently, the natural product group at the Strathclyde Institute for Drug Research (SIDR) in Glasgow has worked with collaborators in Latin America to commercialise anti-anxiety leads that were found by studies on plants used locally as sedative teas. A series of flavonoids was found that acted as partial agonists on some subtypes of benzodiazepine sites in the brain. The most interesting compounds could act like benzodiazepines to reduce behaviour associated with anxiety, but without causing sedation or memory impairment, which are common side effects of benzodiazepines (Medina et al., 1990, 1997; Marder and Paladini, 2002).

In another example, a traditionally used topical preparation from the flowers of *Calendula officinalis* was subjected to bioassay-guided fractionation to try to isolate compounds that might be responsible for the effectiveness of the natural product extract in treating patients with psoriasis. Several related compounds were defined as being cytostatic, rather than cytotoxic, to skin cells in culture (US Patent, 2001).

High Throughput Screening Random Screening and Natural Products

Natural products are a superbly diverse chemical collection that can be productively applied to random screening approaches to drug discovery. This requires natural products researchers to be aware of differences between traditional and modern approaches to drug discovery (Harvey 1998).

Modern drug discovery is dominated by molecular approaches, e.g., with cloned receptors and high throughput screening (HTS). Although molecular strategies also include so-called rational drug design, random screening is probably the most prevalent activity, and also the most relevant for natural products researchers. Random screening is likely to be more productive than other approaches because of the continuing technical challenges faced by the other technologies. For example, with rational drug design, accurate three-dimensional information about the drug's binding target is needed. Molecular biology gives an abundance of predicted protein sequence data, but potential therapeutic targets are not linear proteins. Therefore, structural information is required, but currently, X-ray crystallography is too slow to keep pace with the production of sequence information or not feasible because the protein targets are integral membrane proteins, and *de novo* prediction of structures from linear protein sequences is still an inexact art.

Random screening is the rapid testing of large numbers of compounds on molecular targets—often referred to as *'high throughput screening'* (or HTS). It is built on molecular biology to provide the assays and robotics and IT to provide the throughput (e.g., up to 500,000 points/week/assay). However, the success of any HTS programme depends on the chemical diversity that is applied to the assay. This is because success depends on detecting a specific binding interaction between the target and one of the test compounds: the greater the variations in the three-dimensional shapes of the compounds and in their chemical characteristics, the greater the chance of a detectable interaction with the target. Compound supply, therefore, becomes critical for successful HTS programmes, and three-dimensional diversity of compounds is even more important than the number of compounds.

Compounds for HTS can come from two main sources: natural products and synthetic chemistry. Some large collections of synthetic chemicals are available, e.g., from years of in-house synthesis in major pharmaceutical companies, or from collections of the output of many academic laboratories. However, these numbers are still small when the HTS system can assay half a million compounds each week. Due to this,

many companies have turned to synthetic chemistry based on automated parallel syntheses, using so-called combinatorial chemistry. These methods can provide very high numbers of compounds, but they are generally lacking in three-dimensional diversity. Combinatorial chemistry also suffers from a number of practical issues relating, for example, to the purity of the screening samples, to their stability, and to the reproducibility of the syntheses on scale-up. Few, if any, drugs have reached the market based on initial hits from combinatorial libraries (Newman et al., 2003).

In contrast, natural products offer much greater structural diversity. In a comparison of published chemical databases, 40% of chemical scaffolds from natural products were found to be absent from synthetic libraries (Henkel et al., 1999). An analysis of the properties of a combinatorial chemistry collection, natural products and drug molecules indicated that the synthetic compounds provided relatively little chemical diversity compared to that of drugs and natural products (Feher and Schmidt, 2003). Additionally, very few of the world's natural products have been tested in HTS programmes (Harvey and Waterman, 1998, Harvey, 2000).

What holds back the use of natural products in high throughput screening?

Despite their chemical diversity, natural products are not favoured in HTS because of several real and perceived difficulties. These include their chemical complexity; the difficulty of screening mixtures of compounds in natural product extracts; the time-consuming nature of natural products chemistry; the belief that screening of natural products gives rise to large numbers of artefacts; the supposedly common occurrence of synergistic actions between different components in an extract; the fear of poor reproducibility between different batches of extracts, possibly from seasonal effects on plant secondary metabolism; the uncertainty of being able to obtain resupplies of an interesting extract in large quantities; and the general political problems of access to biodiversity and the implications of the United Nations Convention on Biological Diversity (CBD) (Harvey, 2001).

Given the continued successes deriving from natural product lead compounds (several of the world's current top 20 most valuable drugs are natural products or derived from a natural product lead; Newman et al., 2003; Butler, 2005), it seems worthwhile for the screening departments of pharmaceutical companies to consider natural products as a source of compounds for screening. The structural diversity is acknowledged to be high, so can the other technical barriers be reduced?

Some of the comparisons of the analysis of databases of structures of

natural products and synthetic compounds indicate that natural products are not necessarily much more complex chemicals than the synthetic ones (Henkel et al., 1999). Also, there have been many advances in separation chemistry and in techniques for analysis and structural elucidation of natural products (Bindseil, 2001; Wolfender, 2001; Cremin and Zeng, 2002; Eldridge et al., 2002; Hu et al., 2005). Therefore, following up initial hits made with extracts is not necessarily any longer or more difficult than scaling up and reconfirming hits made from a combinatorial library. Additionally, the higher resolution of current techniques means that structures can be obtained from much smaller quantities of natural products than before (Hu et al., 2005), opening the way to early production of synthetic material and of analogues for optimisation studies.

Another perceived hindrance to the wider use of natural products in HTS systems is the difficulty of access to biodiversity. Most companies with interests in screening natural products are aware of the CBD, but few companies are very clear on its impact. The CBD has been ratified by most countries in the world in recognition of a need to encourage the preservation of the world's biodiversity. In addition to its conservation purposes, the CBD provides a framework for the sustainable exploitation of the genetic resources contained in biodiversity. Under the CBD, countries are recognised as having sovereign rights over the biodiversity within their national boundaries. Countries have to develop appropriate regulations to facilitate access to biodiversity for research and development purposes. Access has to be under principles of prior informed consent and to involve fair and equitable benefit-sharing. Involvement of source countries in research on their biodiversity and technology transfer to the bioresource-rich countries are expected under the CBD.

This raises many practical problems for companies wishing to access samples of natural products for screening. Although many countries have ratified the CBD several years ago, very few have introduced the necessary laws and regulations governing access. Also, it is daunting to companies seeking the broadest range of biodiversity because they would need to establish links with several different countries throughout the world.

Conversely, it is also daunting for research groups working on traditional medicinal leads to know how to gain additional value from natural products that are no longer actively being pursued in the traditional medicines validation programme. Natural product researchers may not find it easy to make appropriate contacts with counterparts in HTS companies. Also, the numbers of samples that they can contribute may be too small to interest a company.

For these reasons, it would be useful for groups of natural products researchers in different countries to operate in networks that pool their

resources. If such networks also have a single contact for commercialisation of natural products available for random screening, they will facilitate the development of collaborations with industry.

SIDR's natural product network

An example of such a collaborative research network is that organised by SIDR. It is long-established, being operative for more than 10 years and self-sustaining through commercial interactions. The network was originally based on research contacts between the Phytochemistry Research Group of the University of Strathclyde and several departments and institutes in developing countries. The network is based on a collaborative approach to natural product-based drug research, wherever possible. Each institution in the network signs a legally binding agreement with the University of Strathclyde covering conditions of collecting (sustainable, no endangered species, compatible with local laws and regulations) and a commitment to training, technology transfer and involvement in research projects. Benefit sharing from commercial exploitation coordinated by SIDR is on a 60:40 basis, with 60% of income going to the overseas institution.

Currently, groups in 20 different countries are involved. The natural product library that has been created is largely based on higher plants. Due to the geographical spread of the participants, samples from over 90% of the families of higher plants are included in the collection (Figure 2). Such biodiversity guarantees exceptionally high chemical diversity, making the network's library particularly attractive for HTS purposes.

The collection has been used in several screening collaborations with the pharmaceutical industry, as well as for academic purposes. Unique

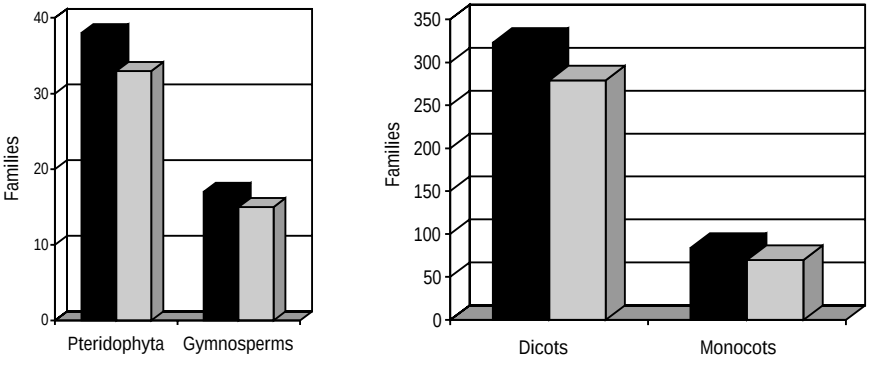

Fig. 2. A comparison of the total plant families (black columns) and families represented in the screening collection of SIDR (light columns).

active molecules have been found against various targets, and these have formed the basis of synthetic programmes aimed at optimising a development candidate. Several routine assays are conducted within SIDR in attempts to find potential lead compounds for future exploitation. The assays are summarised in Table 1.

Table 1. Drug discovery assays in routine use at SIDR

Assay	*Therapeutic area*
Serotonin receptor antagonists	Anti-migraine
Serotonin uptake inhibitors	Anti-depressants
Potassium channel blockers	Immunosuppressants
Tumor necrosis factor blockers	Anti-inflammatory
Cell adhesion inhibitors	Anti-inflammatory/anti-cancer
Tumor cell cytotoxics	Anti-cancer
Tumor enzyme inhibitors	Anti-cancer
Skin cell inhibitors	Anti-psoriasis
Bacterial replication	Antibiotics
Bacterial biofilm inhibitors	Anti-infectives
Glucose uptake promoters	Anti-diabetics
Parasite growth assays	Anti-parasitics
Metabolic enzyme inhibitors	Anti-diabetics
Proteinase inhibitors	Anti-inflammatory and anti-Alzheimer's
Muscarinic receptor agonists	Analgesics
Adenosine receptor antagonists	Neuroprotectives and cognitive enhancers
Antioxidants	Neuroprotectives
Cell growth and nuclear mutations	Genotoxicity and mutagenicity
Cardiac ion channels	Cardiotoxicity

Conclusions and Future Perspectives

From the average numbers of compounds that drop out of industrial drug development during toxicological, pharmacokinetic and clinical testing, it is evident that not all leads from traditionally used natural medicines will succeed as pharmaceutical products. However, natural products can be applied to HTS where their exceptional chemical diversity can provide new lead structures against molecular targets. Despite this, the pharmaceutical industry is not generally enthusiastic about using natural products in HTS systems. The advances in the processing of natural products need to be more widely appreciated, and there is a need for networks of natural

products researchers to provide convenient and politically appropriate access to the world's biodiversity and to engage in modern approaches to drug discovery. This is an opportunity for researchers based in academia to contribute to the discovery of leads for new medicines.

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7 Fungal Endophytes: A Potential Source of Anticancer Compounds

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Introduction

For many years the study of natural products from endophytes has been of low priority for research as it was not known that plants serve as a reservoir for untold number of microbes. The whole scenario of natural product drug discovery programme changed with the discovery of *Taxomyces andreanea*, the producer microorganism of taxol from *Taxus brevifolia* the world's first billion dollar anticancer drug. This change not only helped in conserving the world's diminishing biodiversity but also the price of the drug was reduced as it could be produced via fermentation (Suffness, 1995).

First described by de Bary (1866), endophytes are defined as microorganisms that colonize internal plant tissues. Petrini (1991) defined them as microorganisms that inhabit, at least for one period of their life cycle, plant inner tissues without causing any apparent harm to the host. Hallmann et al. (1997) described them as those that may be isolated from surface sterilized plant parts or extracted from inner tissues and cause no damage to the host plant. It has now been shown by molecular techniques that microorganisms that are not culturable on usual media and conditions can be found inside plants.

The most frequently isolated endophytes are the fungi. Dreyfuss and Chapela (1994) estimated that there may be at least 1 million species of endophytic fungi alone. Fungal endophytes have been reported from mostly

all plants studied, viz., monocots (Alquati, 1999; Pamphile and Azevedo, 2002; Krishnamurthy and Hemalatha, 2003) and dicots (Bettucci et al., 1999; Mahesh et al., 2005) including mangroves (Maria and Sridhar, 2003) and sea grasses (Devrajan et al., 2002). There are reports of endophytes isolated from algae, lichens, moss, ferns, gymnosperm (Carroll and Carroll, 1978; Petrini, 1986; Petrini et al., 1990; Kralj et al., 2006). The majority of fungal endophytes research has been centralized on rhizosphere endophytes with focus on mycorrhizal fungi (Rygiewicz and Andersen, 1994; Redecker et al., 2000). Endophytes also occur in the phyllosphere, criptically inhabiting ariel tissue such as leaves and stems of all plant species examined (Arnold, 2005).

Endophytes produce many substances of pharmaceutical interest, which are originally characteristic of the host. This may be explained by horizontal gene transfer between the endophyte and the host and could occur during the evolutionary process. Chemical compounds produced by endophytes have high commercial value especially for diseases such as cancer, inflammation, diabetes, and bacterial and fungal infections. The success of natural products from endophytes depends upon the diversity of microorganisms.

Collection and selection of samples from diverse ecosystem

Sample collection for isolation of microbes is usually done without definite strategy. The collection programme should take into consideration the biogeography of ecosystem as microbes quickly adapt and respond to the environment in which they survive by generating unique secondary metabolites.

Diverse regions like tropical forests, temperate forests, mangroves, marine algae, sea grasses etc. often generate novel microorganisms, which are the source of novel compounds. Analysis of various ecosystems of a particular region is important. Microorganisms surviving in these unique sites possess unique metabolic pathways to adapt and survive.

Selection of the sample should be done judicially. In case of endophytes, which plant part should be used and why should be kept in mind. For example the bark of neem (*Azadirachta indica*) possess anti-inflammatory, antiarthritic, antipyretic, hypoglycaemic, antigastric ulcer, spermicidal, antifungal, antibacterial, diuretic, antimalarial, immuno-modulatory and antitumor properties (Dahanukar et al., 2000; Mahesh et al., 2005), so the chances of getting novel microorganisms from bark is very high. Hence microbiologists should work in association with botanists and ecologists to maximize the likelihood of finding novel strains and in turn novel compounds.

Anticancer compounds produced by endophytic fungi

Plants have a long history of use in the treatment of various diseases, this is well documented in Ayurveda (Indian medicine system) e.g. in the traditional medicine system the leaves of *Taxus baccata* are used in the treatment of cancer (Hartwell, 1982; Kapoor, 1990). Another example is Madagascar's periwinkle *Catharanthus roseus*, which is used for the treatment of diabetes, and vinca alkaloids, vincristin and visblastin were isolated from this plant (Brossi and Suffness, 1990). Similarly, camptothecine, an anticancer compound was isolated from the Chinese ornamental tree *Campothecea acuminata* (Potmeisel and Pinedo, 1995).

The endophytes, which harbours in the plants, are known to produce various useful bioactive metabolites. Endophytic species such as *Trichoderma, Phoma, Alternaria, Acremonium, Stachybotrytis,* have yielded several 100 bioactive metabolites out of ~ 8600 reported from fungi (Berdy, 2005).

Endophytes produce certain phytochemicals originally characteristic of the host. These substances are synthesized *via* various metabolic pathways e.g. polyketide, isoprenoid, or amino acid derivation, and belong to diverse structural groups, i.e. steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasins (Tan and Zou 2001, Zhang et al. 2006). In the year 1993, Stierle et al. showed that paclitaxel (Taxol) **(1)** (Fig. 1) a complex diterpene used as an antitumor agent was isolated from endophytic fungus *Taxomyces andreanea*, recovered from *Taxus brevifolia*. Another taxol producer was *Pestalotiopsis microspora* isolated from *T. wallachiana* (Strobel et al., 1996). Another example is vincristine **(2)** (Fig. 1), which was isolated from *Catharanthus roseus* along with vinblastine **(3)** (Fig. 1), and they are very useful in the treatment of childhood leukemia (Chabner et al., 1996). Various endophytic fungi viz. *Fusarium oxysporum* 97CG3 and Mycelia sterilia 97CY3 isolated from *Catharanthus roseus* produce vincristine (Zhang et al., 2000; Yang et al., 2004). Similarly camptothecin **(4)** (Fig. 1) can be isolated from various endophytic fungi (Puri et al., 2005). These compounds are normally obtained from the host plant, but these findings showed that microorganisms could also produce these drugs.

After the discovery of taxol from endophytic fungi extensive work has been done to get novel secondary metabolites from higher plants. But less attention was paid to the sources like endophytic fungi from mangroves, ferns, lichens and algae for getting novel secondary metabolite.

Here the metabolites obtained from endophytic fungi of plants, mangroves, and marine algae and their potential as anticancer agent are covered. Many of these compounds are shown in Table 1.

Table 1. Cytotoxic compounds reported from endophytic fungi

Fungus	*Plant source*	*Compound*	*Reference*
From plants			
Entrophospora infrequens	*Nothapodytes foetida*	Camptothecin	Amna et al., 2006
Endophytic fungus (Phycomycetous)	*Nothapodytes foetida*	Camptothecin	Puri et al., 2005
Endophytic fungus	*Camptotheca acuminata*	Camptothecin	Liu and Yu, 2004
Chaetomium globosum IFB-E019	*Imperator cylindrica*	Chaetoglobosin U, C, E, F and Penochalasin A	Ding et al., 2006
Emericella variecolor	*Croton oblongifolius*	Shamixanthone, 14-methoxytajixanthone-25-acetate, Tajixanthone methanoate, and Tajixanthone hydrate	Pornpakakul et al., 2006
Aspergillus niger IFB-E003	*Cynodon dactylon*	Aspernigerin	Shen et al., 2006a
Myrothecium roridum IFB-E009 and *IFB-E012*	*Trachelospermum jasminoides* and *Artemisia annua*	Myrothecines A-C	Shen et al., 2006b
Neoplaconema napellum IFB-E016	*Hopea hainanensis*	Neoplaether	Wang et al., 2006a
Chaetomium globosum	*Ephedra fasciculata*	Globosumones A-C	Bashyal et al., 2005
Pleospora sp. IFB-006	*Imperata cylindrica*	7-methoxy-2-methyl-3,4,5-trihydroxyanthraquinone, Physcion, Macrosporin, Deoxybostrycin, Altersolanol B, Dactylariol	Ge et al., 2005
Microsphaeropsis olivacea	*Pilgerodendron uviferum*	Graphislactone A, Botrallin	Hormazabal et al., 2005

Contd.

Table 1 continued

Fungus	*Plant source*	*Compound*	*Reference*
Cephalosporium acremonium IFB-E007	*Trachelospermum jasminoides*	Graphislactone G, Graphislactone H, Graphislactone A, Alternariol monoethyl ether	Zang et al., 2005
Phomopsis cassiae	*Cassia spectabilis*	Ethyl 2,4-dihydroxy-5,6-dimethyl-benzoate, Phomopsilactone	Silva et al., 2005
Unidentified Endophytic fungus	*Castaniopsis fissa*	Ergosta-8(9),22-diene-3,5,6,7-tetraol (3b,5a,6b,7a,22E) (A)	Li et al., 2004
Aspergillus niger IFB-E003	*Cynodon dactylon*	Rubrofusarin B, Aurasperone A	Song et al., 2004
Mycelia sterilia 97CY3	*Catharanthus roseus*	Vincristine	Yang et al., 2004
Fusarium oxysporum	*Catharanthus roseus*	Vincristine	Zhang et al., 2000
Hormonema dematioides	Pinus species	Hormonemate	Filip et al., 2003
Aspergillus parasiticus	*Sequoia sempervirens*	Sequoiatones C-F, Sequoiamonascins A-D	Stierle et al., 2003
Chaetomium globosum	*Maytenus hookerii*	Chaetoglobosin A	Zhang et al., 2002
Phomopsis sp. BCC 1323.	*Tectona grandis*	Phomoxanthones A and B	Isaka et al., 2001
Unidentified Endophytic fungus	*Ficus microcarpa*	Nomofungin	Ratnayake et al., 2001a
Unidentified Endophytic fungus	*Ficus microcarpa*	Microcarpalide	Ratnayake et al., 2001b
Phomopsis longicolla	*Dicerandra frutescens*,	Dicerandrols A, B and C	Wagenaar et al., 2001
Cytospora sp.	*Conocarpus erecta*	Cytoskyrins A and B	Brady et al., 2000
Rhinocladiella sp.	*Tripterygium wilfordii*	Cytochalasin E, 22-oxa-(12)-cytochalasins 1,2,3	Wagenaar et al., 2000
Acremonium sp.	*Taxus baccata*	Leucinostatin A	Strobel et al., 1997b

Contd.

Table 1 continued

Fungus	*Plant source*	*Compound*	*Reference*
Stegolerium kukenani	*Maguireothamnus speciosus*	Taxol	Strobel et al., 2001
Tubercularia sp. TF5	*Taxus mairei*	Taxol	Wang et al., 2000
Periconia sp.	*Torreya grandifolia*	Taxol	Li et al., 1998
Pestalotiopsis guepinii	*Wollemia nobilis*	Taxol	Strobel et al., 1997a
Pestalotiopsis microspora	*Taxodium distichum*	Taxol	Li et al., 1996
Pestalotiopsis microspora	*Taxus wallachiana*	Taxol	Strobel et al., 1996
Taxomyces andreanea	*Taxus brevifolia.*	Taxol	Stierle et al., 1993
Pestalotiopsis microspora	*Torreya taxifolia*	Torreyanic acid	Lee et al., 1996
From Mangrove			
Endophytic fungus No. 1893	Mangrove	Mycoepoxydiene	Chen et al., 2005
Dothiorella sp. strain HTF3	*Avicennia marina*	Cytosporone B	Xu et al., 2004
Endophytic fungus No. 2524	*Avicennia marina*	[2',3'-dihydroxytetracosanolyamino]-1,3-dihydroxyoctadecane and [2',3'-dihydroxydocosanoylamino]-1,3-dihydroxyoctadecane	Li et al., 2003
Endophytic fungus No. 2526	*Avicennia* sp.	Sterigmatocystin	Zhu et al., 2003
Aigialus parvus BCC 5311	Mangrove	Aigialomycin D	Isaka et al., 2002
Halosarpheia sp. *strain 732*	Mangrove	Enniatin G	Lin et al., 2002
From Marine Algae			
Emericella nidulans var. *acristata*	Green alga	Emindole DA, Arugosin A, Arugosin B	Kralj et al., 2006

Contd.

Table 1 continued

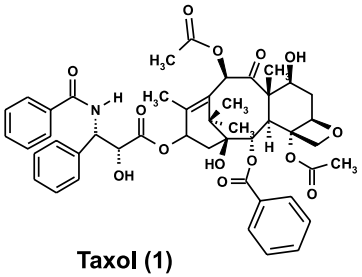
Fungus	*Plant source*	*Compound*	*Reference*
Chaetomium globosum	*Polysiphonia urceolata.*	Chaetopyranin	Wang et al. (2006b)
Apiospora montagnei.	*Polysiphonia violacea*	(+)-epiepoxydon	Klemke et al., 2004
Leptosphaeria sp. OUPS-N80	*Sargassum tortile*	Leptosins A–S	Takahashi et al., 1994a,b, 1995a,b; Yamada et al., 2002, 2004
Trichoderma virens	*Halimeda* sp.	Trichodermamide B	Garo et al., 2003
Scytalidium sp. CNC-310	*Halimeda* sp.	Scytalidamides A, B	Tan et al., 2003
Fusarium chlamydosporum OUPS-N124	*Carpopeltis affinis*	Fusaperazine A	Usami et al., 2002
Fusarium sp.	*Avrainvillea* sp.	N-Methylsansalvamide	Cueto et al., 2000
Penicillium sp.	*Enteromorpha intestinalis*	Penochalasins A-H, Penostatins A–I, Communesins A, B	Numata et al., 1993, 1996; Takahashi et al., 1996; Iwamoto et al., 1998, 1999, 2001
Penicillium sp. CNC-350	*Avrainvillea longicaulis*	11,11'-dideoxyverticillin A and 11'-deoxyverticillin A	Son et al., 1999
Aspergillus versicolor CNC-327	*Penicillus capitatus*	Insulicolide A	Belofsky et al., 1998
Aspergillus insulicola	Marine alga	Insulicolide A	Rahbæk et al., 1997
Penicillium waksmanii	*Saragassum ringgoldianum*	Pyrenocine E	Amagata et al., 1998

From plants

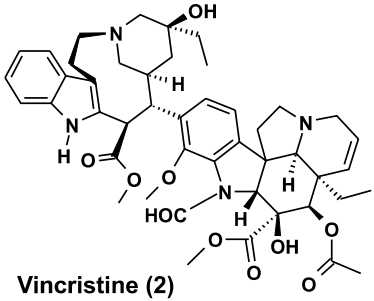
Ding et al. (2006) isolated a new cytotoxic cytochalasan-based alkaloid named chaetoglobosin U **(5)** (Fig. 1), along with four known analogs, chaetoglobosins C **(6)** (Fig. 1), E **(7)** (Fig. 1) and F **(8)** (Fig. 1) and penochalasin A, from the culture of *Chaetomium globosum* IFB-E019, an endophytic fungus residing inside the stem of healthy *Imperata cylindrica*. Chaetoglobosin U exhibited cytotoxic activity against the human nasopharyngeal epidermoid tumor KB cell line with an IC_{50} value of 16.0 μM, comparable to that 14.0 μM of 5-fluorouracil co-assayed as a positive reference (Ding et al., 2006). The four known analogs were moderately active to the cell line, with IC_{50} values of 34.0, 52.0, 48.0, and 40.0 μM, respectively. Another cytochalasan alkaloid chaetoglobosin A **(9)** (Fig. 1) was isolated from endophytic fungus *Chaetomium globosum* of *Maytenus hookerii* (Zhang et al., 2002). Ge et al. (2005) reported seven anthraquinones viz. 7-methoxy-2-methyl-3,4,5-trihydroxyanthraquinone **(10)** (Fig. 1), physcion **(11)** (Fig. 1), macrosporin **(12)** (Fig. 1), deoxybostrycin**(13)** (Fig. 1), altersolanol B **(14)** (Fig. 1) and dactylariol **(15)** (Fig. 1), a new hexahydroanthraquinone named pleospdione **(16)** (Fig. 1) from the culture of *Pleospora* sp. IFB-E006, an endophytic fungus residing in the normal stem of *Imperata cylindrica*. Compounds deoxybostrycin, altersolanol B and dactylariol exhibited significant cytotoxic activity against human colon cancer with IC_{50} of 5.8, 3.3, 0.8, μg/ml (SW1116) and 3.1, 3.7, 1.3 μg/ml agaist leukemia (K562) cell lines respectively while compounds 7-methoxy-2-methyl-3,4,5-trihydroxyanthraquinone, physcion and pleospdione were only weakly or moderately active with IC_{50} of > 100, > 100, 46.2, μg/ml against SW1116 and 100, > 100, 58.8 μg/ml against, K562 cell lines respectively. The compound 5-fluorouracil a positive reference exhibited the IC_{50} of 6.0 and 6.5 μg/ml against SW1116 and K562 cell lines respectively.

Amna et al. (2006) reported *Entrophospora infrequens* isolated from the inner bark tissue of the *Nothapodytes foetida* plant growing in the Jammu region of the state of Jammu and Kashmir, India, was found to produce detectable quantities of camptothecin and its derivatives. Puri et al. (2005) also reported camptothecin from an endophytic fungus belonging to the family Phycomycetes isolated from *Nothapodytes foetida* growing in the western coast of India. It was identified by means of chromatographic and spectroscopic methods and was also compared with an authentic sample for its biological activity against a number of human cancer cell lines. Camptothecin was also reported from various endophytic fungi isolated from *Campotheca acuminata* and it inhibited the growth of HL-60 cells *in vitro* (Liu and Yu, 2004).

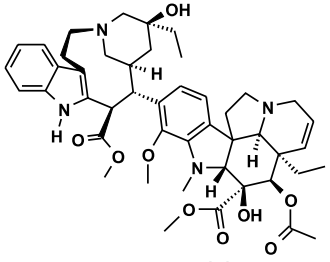
Four xanthones, shamixanthone **(17)** (Fig. 1), 14-methoxytajixanthone-25-acetate, tajixanthone methanoate, and tajixanthone hydrate were



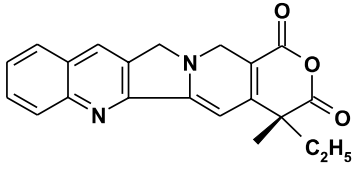
Taxol (1)



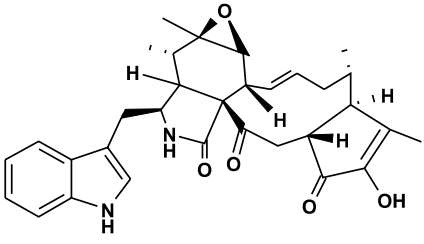
Vincristine (2)



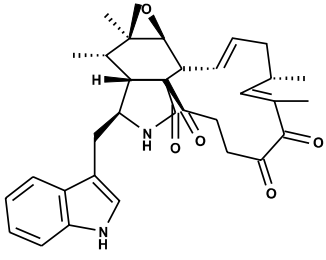
Vinblastine (3)



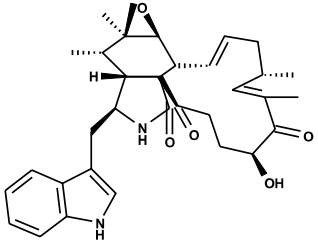
Camptothecin (4)



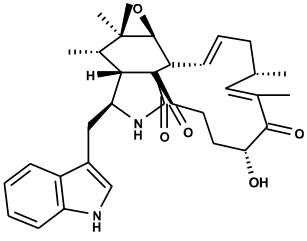
Chaetoglobosin U (5)



Chaetoglobosin C (6)

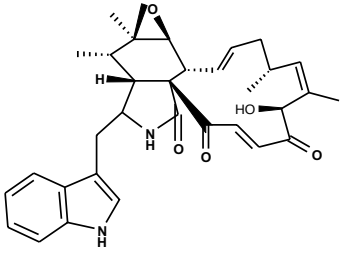


Chaetoglobosin E (7)

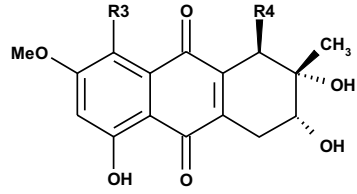


Chaetoglobosin F (8)

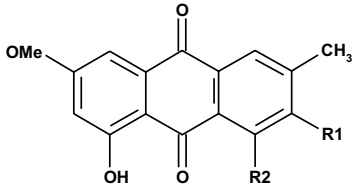
Fig. 1. Structures of cytotoxic metabolites isolated from endophytic fungi.



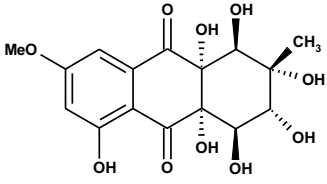
Chaetoglobosin A (9)



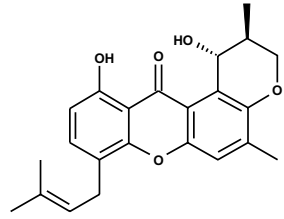
Deoxybostyrin = R3=OH,R4=H (13)
Altersolanol = R3=R4=H (14)
Dactylariol= R3=H, R4=OH (15)



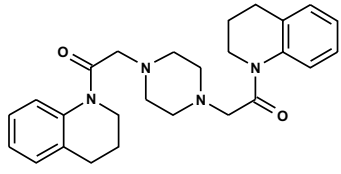
7-methoxy-2-methyl-3,4,5-trihydroxyanthraquinone =R1=R2=OH (10)
Physcion= R1=H,R2=OH (11)
Macrosporin=R1= OH R2=H (12)



Pleospdione (16)



Shamixanthone (17)



Aspernigerin (18)

Fig. 1 continued. Structures of cytotoxic metabolites isolated from endophytic fungi.

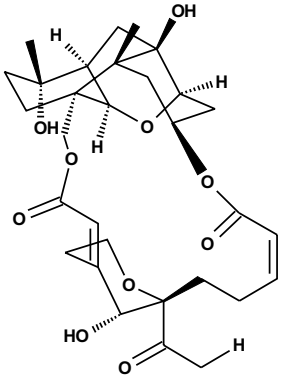
isolated from mycelia of *Emericella variecolor*, an endophytic fungus isolated from the leaves of *Croton oblongifolius* (Pornpakakul et al., 2006). All compounds were tested for cytotoxic activity against various human tumor cell lines including gastric carcinoma, colon carcinoma, breast carcinoma, human hepatocarcinoma, and lung carcinoma. Compounds shamixanthone, 14-methoxytajixanthone-25-acetate and tajixanthone methanoate showed moderate activity with IC_{50} of 15.0, 11.5 and 20.0 mM (KATO3—gastric carcinoma); 21.4, 14.4 and 19.2 mM (SW 620—colon carcinoma); 12.5, 12.1 and 14.1 mM (BT 474—human breast carcinoma) respectively. Only tajixanthone hydrate exhibited moderate activity against all cell lines tested with IC_{50} of 10.9 mM (KATO3—gastric carcinoma) 13.6 mM (SW 620—colon carcinoma), 12.3 mM (BT 474—human breast carcinoma), 16.4 (HEP-G2—human liver hepatoblastoma) and 11.6 mM (CHAGO—human lung carcinoma) respectively.

Shen et al. (2006a) isolated aspernigerin **(18)** (Fig. 1) a novel cytotoxic alkaloid from the extract of a culture of *Aspergillus niger* IFB-E003, an endophyte in *Cynodon dactylon*. Aspernigerin was cytotoxic to the tumor cell lines like nasopharynyeal epidermoid KB, cervical carcinoma HeLa and colorectal carcinoma SW1116 with corresponding IC_{50} values of 22, 46, and 35 μM, respectively.

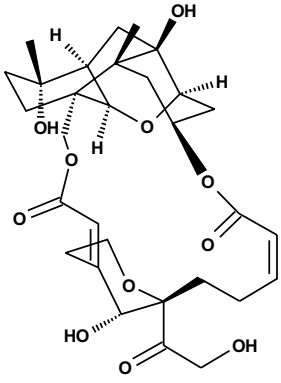
Shen et al. (2006b) isolated three novel cytotoxic 10,13-cyclotrichothecane-derived macrolides, Myrothecines A-C **(19-21)** (Fig. 2) from *Myrothecium roridum* IFB-E009 and IFB-E012, isolated from endophytic fungi found on the two traditional Chinese medicinal plants *Trachelospermum jasminoides* and *Artemisia annua*, respectively.

Wang et al. (2006a) isolated a new diphenyl ether, neoplaether **(22)** (Fig. 2), together with five known compounds viz. monomethylsulochrin, physcion, helvolic acid, ergosterol and ergosterol peroxide, from the culture of *Neoplaconema napellum* IFB-E016, an endophytic fungus residing in the healthy leaves of *Hopea hainanensis*. Neoplaether exhibited significant cytotoxic activity against the human nasopharyngeal epidermoid tumor KB cell line, with an IC_{50} value of 13.0 μg/ml, comparable to that of 5-fluorouracil (2.5 μg/mL) co-assayed as a positive reference.

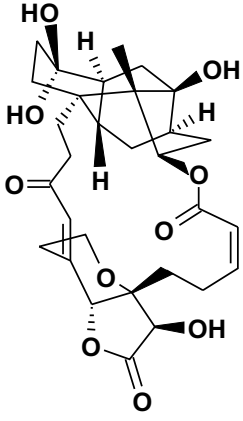
Three new esters of orsellinic acid, globosumones A-C **(23-25)** (Fig. 2), and three known compounds orsellinic acid, orcinol, and trichodion, were isolated from *Chaetomium globosum* endophytic on *Ephedra fasciculata*. All compounds were evaluated for inhibition of cell proliferation in a panel of four cancer cell lines namely NCI-H460 (non-small cell lung cancer), MCF-7 (breast cancer), SF-268 (CNS glioma), and MIA Pa Ca-2 (pancreatic carcinoma), and WI-38 (normal human fibroblast cells). Only globosumones A and B were found to be moderately active. Globosumones A showed the activity with IC_{50} of 6.50, 21.30, 8.80, 10.60 and 13.00 μM



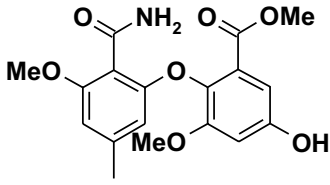
Myrothecine A (19)



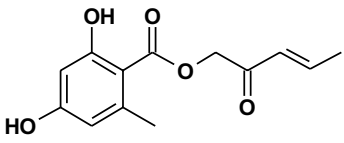
Myrothecine B (20)



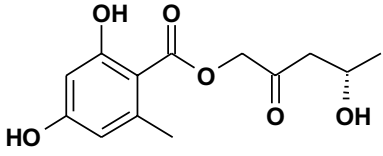
Myrothecine C (21)



Neoplaether (22)

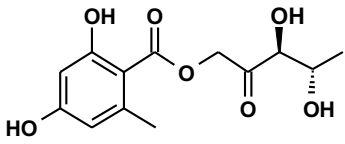


Globosumone A (23)

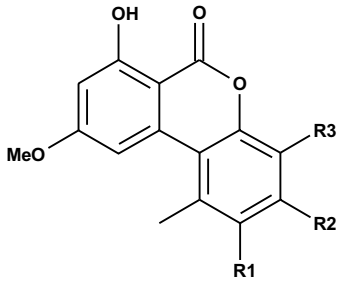


Globosumone B (24)

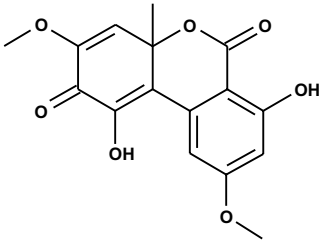
Fig. 2. Structures of cytotoxic metabolites isolated from endophytic fungi.



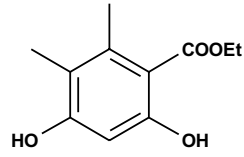
Globosumone C (25)



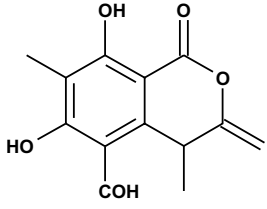
Graphislactone G R1= Cl, R2= OCH3, R3= H (26)
Graphislactone H R1= H, R2= OCH3, R3= OCH3 (27)
Graphislactone A R1= H, R2=OCH3, R3=OH (28)
Alternariol monoethyl ether R1= H, R2=OH, R3= H (29)



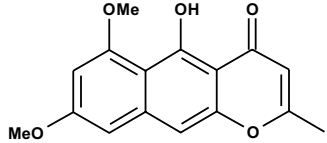
Botrallin (30)



Ethyl 2,4-dihydroxy-5,6-dimethyl benzoate (31)



Phomopsilactone (32)



Rubrofusarin B (33)

Fig. 2 continued. Structures of cytotoxic metabolites isolated from endophytic fungi.

against NCI-H460, MCF 7, SF268, MIA PACa-2 and WI38 cell lines respectively while Globosumones B showed the activity with IC_{50} of 24.80, 21.90, 29.10, 30.20, and 14.20 µM against NCI-H460, MCF 7, SF268, MIA PACa-2 and WI38 cell lines respectively. The doxorubicin a known cytotoxic compound exihibited the IC_{50} of 0.01, 0.07, 0.04, 0.05 and 0.03 µM against NCI-H460, MCF 7, SF268, MIA PACa-2 and WI38 cell lines respectively. (Bashyal et al. 2005).

Two novel 6H-dibenzo[b,d]pyran-6-one derivatives, graphislactone G **(26)** (Fig. 2) and graphislactone H **(27)** (Fig. 2), besides graphislactone A **(28)** and alternariol monomethyl ether **(29)** (Fig. 2) were isolated from the culture of *Cephalosporium acremonium* IFB-E007, an endophytic fungus in *Trachelospermum jasminoides.* The structures of graphislactone G and graphislactone H were established as 2-chloro-7-hydroxy-3,9-dimethoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one and 7-hydroxy-3,4,9-trimethoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one, respectively. Graphislactone G was unique in its bearing a Cl-atom. Anticancer tests showed that all four compounds had pronounced activities against SW1116 cell with IC_{50} values of 21, 12, 8.5, and 14 µg/ml, respectively (Zhang et al., 2005). Similarly, Hormazabal et al. (2005), reported graphislactone A along with botrallin **(30)** (Fig. 2) from *Microsphaeropsis olivacea*, an endophytic fungus of *Pilgerodendron uviferum.* These compounds presented a moderate activity towards AChE, with IC_{50} of 8.1 and 6.1 µg/ml respectively. The cytotoxicity of both compounds was > 1000 and 330 µM, respectively.

Silva et al. (2005) reported two new metabolites, ethyl 2,4-dihydroxy-5,6-dimethylbenzoate **(31)** (Fig. 2) and phomopsilactone **(32)** (Fig. 2) along with known compound 2-hydroxyphenylacetic acid from *Phomopsis cassiae*, an endophytic fungus in *Cassia spectabilis.* Cytotoxicity of these compound were tested against human cervical tumor cell line (HeLa), in *in vitro* assays. Phomopsilactone exhibited weak toxicity (IC_{50} 200 µmol/L) and 2-hydroxyphenylacetic acid a strong toxicity (IC_{50} 10 µmol/L). Cisplatin, a cytotoxic agent was used as positive control with IC_{50} 5 µmol/L.

Li et al. (2004) reported a new sterol, ergosta-8(9),22-diene-3,5,6,7-tetraol(3β,5α,6β,7α,22E) from the mycelia of an unidentified endophytic fungus from *Castaniopsis fissa*. The compound exhibited potent selective cytotoxicity against Bel-7402, NCI 4460 and L-02 cell lines with IC_{50} values 8.445, 5.03 and 13.621 µg/ml, respectively.

Fractionation of the extract of *Aspergillus niger* IFB-E003, an endophyte in *Cynodon dactylon*, gave four known compounds naphtho-gamma-pyrones: rubrofusarin B **(33)** (Fig. 2), fonsecinone A, asperpyrone B and aurasperone A **(34)** (Fig 3). Rubrofusarin B was cytotoxic to the colon cancer cell line SW1116 (IC_{50} 4.5 µg/ml), and aurasperone A inhibitory on XO (xanthine oxidase) (IC_{50} 10.9 µM/l) (Song et al., 2004).

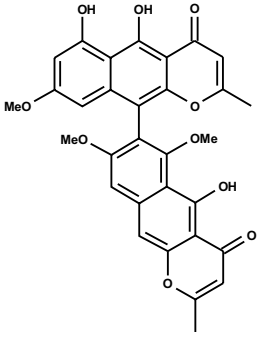
Filip et al. (2003), reported hormonemate **(35)** (Fig. 3). a new cytotoxic and apoptosis-inducing compound from the endophytic fungus *Hormonema dematioides* isolated from living needles of a Pinus species. The compound exhibited cytotoxic effects against the human colon tumor cell lines COLO-320, DLD-1, HT-29, JURKAT, MCF-7 and HL-60 with IC_{50} value of 3.5-7.5 µg/ml., HEP-G2, and HeLa S3 cell were less sensitive with an IC_{50} of 9-10 µg/ml. It also induced apoptosis in COLO-320 cells as detected by a caspase-activity assay and it triggered morphological and physiological differentiation of HL-60 cells into granulocytes, which subsequently died by apoptosis.

Stierle et al. (2003), reported *Aspergillus parasiticus*, a fungal isolate from the bark of a redwood tree (*Sequoia sempervirens*), that can produce the antitumor metabolites sequoiatones C-F **(36-39)** (Fig. 3). Another series of compounds with a new carbon skeleton, the Sequoiamonascins were also isolated. Sequoiamonascins A-D **(40-43)** (Fig. 3) shows moderate activities against cancer cell lines, including MCF7 (breast), NCI-H460 (lung), and SF-268 (CNS).

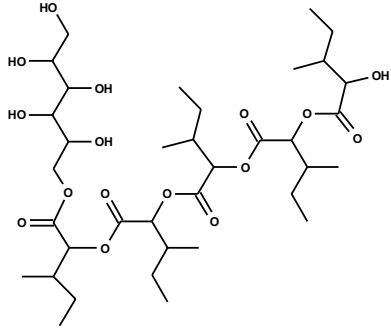
Isaka et al. (2001) reported phomoxanthones A and B **(44-45)** (Fig. 3), two xanthone dimers from the endophytic fungus *Phomopsis* sp. BCC 1323 isolated from *Tectona grandis* from North Thailand. Phomoxanthones A and B exhibited significant cytotoxicity against KB and BC1 cell lines with IC_{50} of 0.99 and 4.1 µg/ml against Kb cells and 0.51 and 0.70 µg/ml against BC1 respectively. The IC_{50} of standard compound ellipticine was 4.6 and 0.6 µg/ml against KB and BC1 respectively.

A new alkaloid nomofungin **(46)** (Fig. 3) was isolated from the fermented broth of an unidentified endophytic fungus obtained from the bark of *Ficus microcarpa*. It disrupts microfilaments in cultured mammalian cells and is moderately cytotoxic, with MICs of 2 and 4.5 µg/mL against LoVo and KB cells, respectively (Ratnayake et al., 2001a). Similarly, a new alkyl-substituted nonenolide microcarpalide **(47)** (Fig. 2) was isolated from fermented broths of an unidentified endophytic fungus isolated from *Ficus microcarpa*. Microcarpalide is weakly cytotoxic to mammalian cells with IC_{50} value in the KB (human nasopharyngeal carcinoma) and LoVo (human colon adenocarcinoma) cell lines of 50 and 90 µg/mL respectively (Ratnayake et al., 2001b).

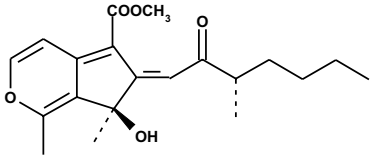
Wagenaar et al. (2001) isolated cytotoxic compounds, dicerandrols A, B, and C **(48-50)** (Fig. 3) from *Phomopsis longicolla*, an endophytic fungus of the endangered mint *Dicerandra frutescens.* Extensive NMR and HRFABMS experiments identified these new yellow antibiotic and cytotoxic compounds as 2,2'-dimeric tetrahydroxanthones. These compounds exhibited cytotoxic activity against A549 and HCT 116 cell lines with IC_{100} of 7.0, 1.8 and 1.8 against A549 and 7.0, 1.8 and 7.0 against HCT 116 cell lines respectively.



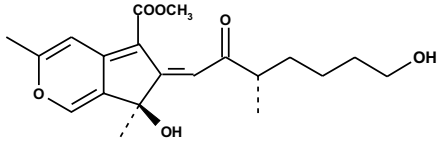
Aurasperone A (34)



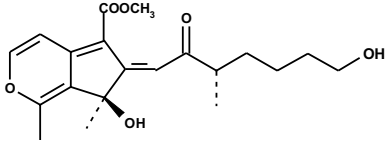
Hormonemate (35)



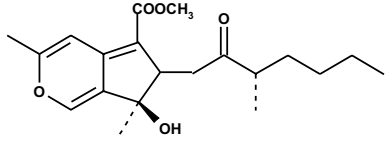
Sequoiatone C (36)



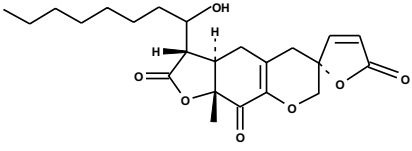
Sequoiatone D (37)



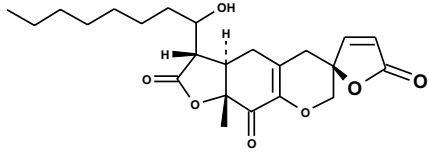
Sequoiatone E (38)



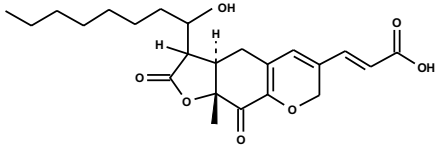
Sequoiatone F (39)



Sequoiamonascin A (40)

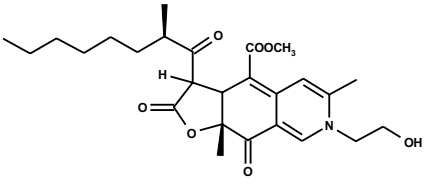


Sequoiamonascin B (41)

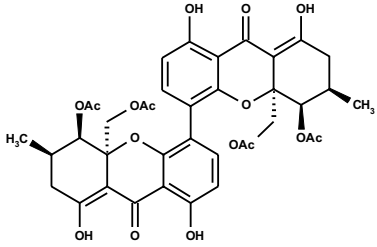


Sequoiamonascin C (42)

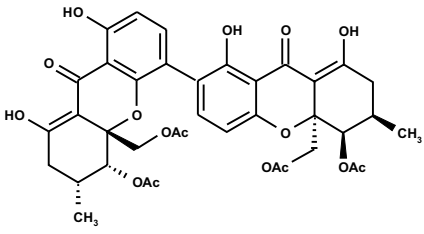
Fig. 3. Structures of cytotoxic metabolites isolated from endophytic fungi.



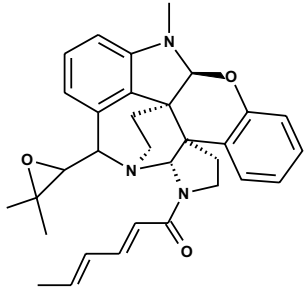
Sequoiamonascin D (43)



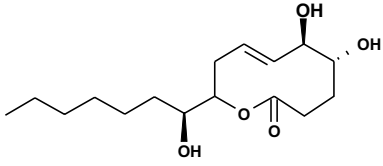
Phomoxanthone A (44)



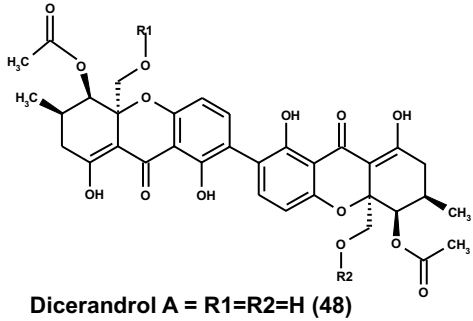
Phomoxanthone B (45)



Nomofungin (46)



Microcarpalide (47)



Dicerandrol A = R1=R2=H (48)
Dicerandrol B = R1=Ac, R2=H (49)
Dicerandrol C = R1=R2= Ac (50)

Fig. 3 continued. Structures of cytotoxic metabolites isolated from endophytic fungi.

Brady et al. (2000) isolated cytoskyrins A **(51)** (Fig. 4) and B, new BIA active bisanthraquinones from endophytic fungus *Cytospora* isolated from the branch of a *Conocarpus erecta*. Cytoskyrin A (Fig. 2) exhibited poor cytotoxicity against tumor cell lines (IC_{50} > 5 µg/ml) as compared to known antitumor agents.

Wagenaar et al. (2000) reported cytochalasin E and three new cytotoxic cytochalasins from a culture of the endophytic fungus *Rhinocladiella* sp. isolated from *Tripterygium wilfordii* using bioassay-guided fractionation. Extensive NMR and HRCIMS experiments identified these new compounds as 22-oxa-[12]-cytochalasins1,2,3. **(52-54)** (Fig. 4) Cytochalasin E **(55)** (Fig. 4) is significantly more potent (15-100 fold) against all cell lines tested i.e. A2780S, SW-620, HCT-116. 22-oxa-[12]-cytochalasins1,2,3 did not show any selectivity in the cell lines tested.

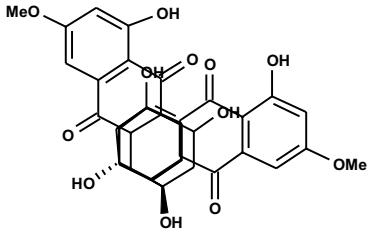
Strobel et al. (1997a) isolated *Pestalotiopsis guepinii*, endophyte from *Wollemia nobilis*, an araucariaceous plant, which produces taxol. Wang et al. (2000) reported taxol-producing endophytic fungus *Tubercularia* sp TF5, isolated from *Taxus mairei*. The fungal taxol, had strong cytotoxic effect towards KB and P388 cancer cell lines, and the ID_{50} values of cytotoxicity was about 1/2400 for KB cells and 1/38400 for P388 cells *in vitro*, tested by MTT assay. It also enhanced microtubule stability, bundling in culture cells and induced tubulin polymerization *in vitro* (1/100 v/v) similar to the authentic taxol (25 µg/ml). The other producers of taxol are *Pestalotiopsis microspora* isolated from *Taxodium distichum* (Li et al., 1996); *Periconia* sp. isolated from *Torreya grandifolia*, (Li et al., 1998); *Stegolerium kukenani* isolated from *Maguireothamnus speciosus* (Strobel et al., 2001).

Acremonium sp. occurs as an endophyte in European yew (*Taxus baccata*), which produces a series of peptide antifungal, anticancer agents known as the leucinostatins. Leucinostatin A **(56)** (Fig. 4) possessed activity against certain human cancer cell lines, for instance, its IC_{50} value is 2.3 nM for breast cancer cell line BT-20 contrasted with 640 nM for a normal mammary cell line (Strobel et al. 1997b).

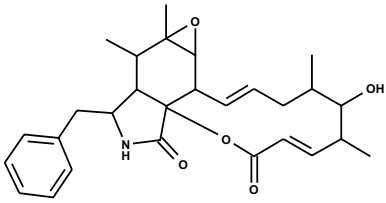
Lee et al. (1996) isolated torreyanic acid **(57)** (Fig. 4), a selectively cytotoxic quinone dimer from the endophytic fungus *Pestalotiopsis microspora* isolated from *Torreya taxifolia.* Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5 to 10 times more potent cytotoxicity in cancer cell lines that are sensitive to protein kinase C agonists; it causes cell death by apoptosis.

From mangroves

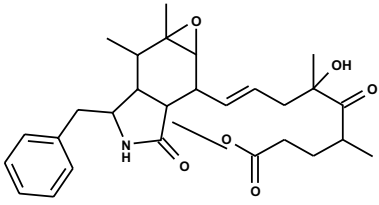
Chen et al. (2005) isolated mycoepoxydiene **(58)** (Fig. 4), which showed cytotoxicity toward human tumor cells *in vitro*, from the fermented broth



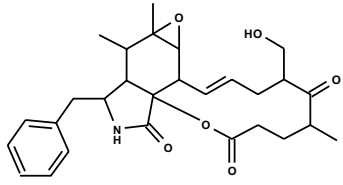
Cytoskyrin A (51)



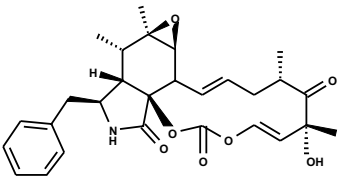
22-oxa-[12]-cytochalasin1 (52)



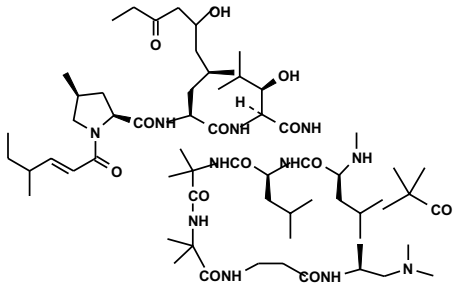
22-oxa-[12]-cytochalasin 2 (53)



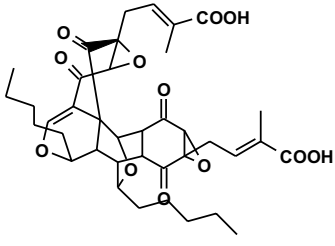
22-oxa-[12]-cytochalasin 3 (54)



Cytochalasin E (55)

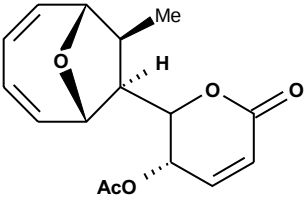


Leucinostatin A (56)

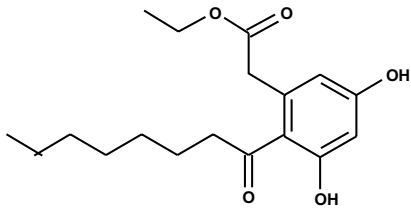


Torreyanic acid (57)

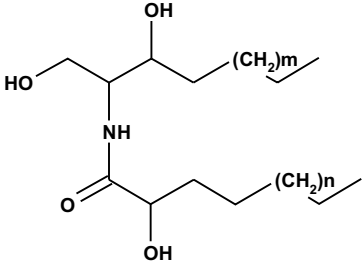
Fig. 4. Structures of cytotoxic metabolites isolated from endophytic fungi.



Mycoepoxydiene (58)

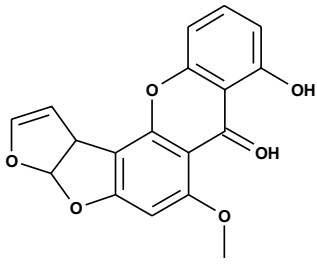


Cytosporone B (59)

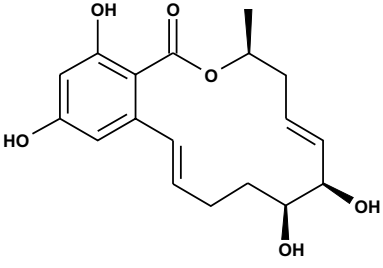


[2',3'-dihydroxytetracosanolyamino]-1, 3-dihydroxyoctadecane = m=12, n=18 (60)

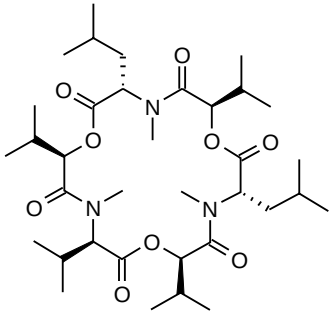
[2',3'-dihydroxydocosanolyamino]-1, 3-dihydroxyoctadecane = m=12, n=16 (61)



Sterigmocystin (62)



Aigialomycin D (63)



Enniatin G (64)

Fig. 4 continued. Structures of cytotoxic metabolites isolated from endophytic fungi.

of the marine mangrove endophytic fungus No. 1893. It shows high antitumor activity with IC_{50} of < 6.25 µg/ml against KB cell lines (Lin et al., 2005) and 5.5 µg/ml against HeLa cell lines (Chen et al., 2006) respectively.

Xu et al. (2004) reported cytosporone B **(59)** (Fig. 4)**,** isolated from an endophytic fungus, *Dothiorella* sp. strain HTF3 isolated from the mangrove plant *Avicennia marina* at the estuary of Jiulong River, Fujian Province. Its IC_{50} values were 4 µg/ml and 0.062 5 µg/ml against human epidermal carcinoma of oral cavity, KB cell line and human Burkitt's lymphoma, Raji cell line, respectively.

Li et al. (2003), reported two new ceramides, [2',3'-dihydroxytetracosanolyamino]-1,3-dihydroxyoctadecane **(60)** (Fig. 4) and [2',3'-dihydroxydocosanoylamino]-1,3-dihydroxyoctadecane **(61)** (Fig. 4), from the culture extract of an unidentified endophytic fungus No. 2524 isolated from the seed of mangrove *Avicennia marina* of Hong Kong. These ceramides did not exhibit significant cytotoxicity against the human cancer cell lines Bel-7402 and NCI-4460 and the normal human cell line L-02 in preliminary cytotoxic activity investigation.

Zhu et al. (2003) isolated sterigmatocystin **(62)** (Fig. 4), from endophytic fungus No. 2526 of mangrove *Avicennia* from the South China Sea, which showed a weak cytotoxic activity against Bel-7402 and NCIH-460 with with IC_{50} of 96.53 and 72. 52 µg/ml respectively.

Isaka et al. (2002) isolated Aigialomycins A, B, C, D, E, new 14-membered resorcylic macrolides together with known hypothemycin from the mangrove fungus, *Aigialus parvus* BCC 5311. Only Aigialomycin D **(63)** (Fig. 4)**,** have cytotoxic and antiplasmodial activity. Hypothemycin and Aigialomycins A, B, C, D, E exhibited cytotoxic activity with IC_{50} of 17, >20, > 20, >20, 6.6 and > 20 µg/ml against KB (Human epidermoid carcinoma) cell lines and 6.2, 11, >20, > 20, 18 and 15 µg/ml against BC1 (human breast cancer cells) respectively.

Lin et al. (2002) isolated novel cyclic depsipeptide, enniatin G **(64)** (Fig. 4), together with enniatins B and B4 from the mangrove fungus *Halosarpheia* sp. strain 732 from the South China Sea. Enniatin G exhibited activity against Heps 7402, ED_{50} 12mg/ml.

From marine algae

Kralj et al. (2006) isolated *Emericella nidulans* var. *acristata* from a Mediterranean green alga. Cultivation of this fungus yielded emindole DA**(65)** (Fig. 5), the indole alkaloid, which displayed antitumor activity in a panel of 36 human tumor cell lines, exhibiting a mean IC_{50} value of 5.5 µg/ml in an *in vitro* survival and proliferation assay. Arugosin A **(66)** (Fig.

3) and arugosin B **(67)** (Fig. 5) were coproduced in the culture, which showed moderate antitumor activity toward individual tumor cell lines.

Chaetomium globosum, an endophytic fungus which was isolated from the inner tissue of the marine red alga *Polysiphonia urceolata*, resulted in the isolation of a new benzaldehyde secondary metabolite chaetopyranin **(68)** (Fig. 5) which exhibited moderate to weak cytotoxic activities against three tumor cell lines, with IC_{50} values of 15.4 (human microvascular endothelial cells, HMEC), 28.5 (hepatocellular carcinoma cells, SMMC-7721) and 39.1 µg/ml (human lung epithelial cells, A549) (Wang et al., 2006b).

Klemke et al. (2004) isolated marine fungus *Apiospora montagnei* from the inner tissue of the North Sea alga *Polysiphonia violacea* and reported (+)-epiepoxydon **(69)** (Fig. 5) which exhibited 50% reduction of the initial cell number (LC_{50}) at concentration of 3.6, >10, and 10 µg/ml towards human cancer cell lines HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma) and MCF7 (breast adenocarcinoma) respectively.

Leptosphaeria sp. OUPS-N80 (formerly OUPS-4) isolated from the brown alga *Sargassum tortile* is a prolific fungus and 24 new compounds, leptosins A **(70)** (Fig. 5) and B (–S), have been obtained from this strain (Takahashi et al., 1994a, 1994b, 1995a, 1995b; Yamada et al., 2002, 2004). Leptosins showed strong cytotoxicity against P388 with ED_{50} values in the ng order.

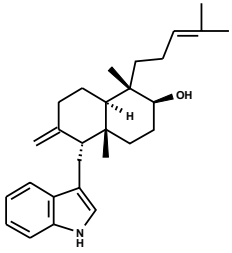
Trichodermamides A and B **(71)** (Fig. 5) two modified dipeptides have been isolated from cultures of the marine-derived fungus *Trichoderma virens.* Trichodermamide B displayed significant *in vitro* cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} of 0.32 µg/ml (Garo et al., 2003).

Scytalidamides A **(72)** (Fig. 5) and B the cytotoxic compounds were isolated from *Scytalidium* sp. CNC-310 isolated from marine alga *Halimeda* sp. collected from Bahamas (Tan et al., 2003). Scytalidamides A and B showed moderate *in vitro* cytotoxicity toward HCT-116 human colon adenocarcinoma with IC_{50} values of 2.7 and 11.0 µM, respectively.

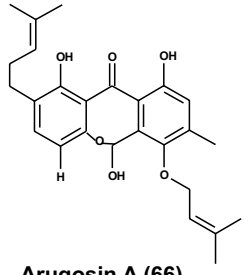
Usami et al. (2002) isolated two new sulphur-containing dioxopiperazine derivatives designated Fusaperazines A and B **(73-74)** (Fig. 5) from *Fusarium chlamydosporum* OUPS-N124 from the red alga *Carpopeltis affinis* of Japan. Fusaperazine A exhibited weak cytotoxic activity against the lymphocytic leukemia cell line P388 (ED_{50} = 22.8 µg/ml).

Cueto et al. (2000) reported *N*-methylsansalvamide **(75)** (Fig. 5) from *Fusarium* sp. which was isolated from the green alga *Avrainvillea* sp. *N*-methylsansalvamide showed moderate cytotoxicity (GI_{50} = 8.3 µM) in the NCI's human tumor cell line screen.

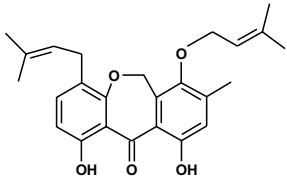
Penicillium sp. OUPS-N79 isolated from the green alga *Enteromorpha*



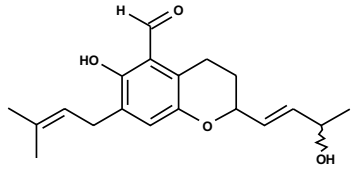
Emindole DA (65)



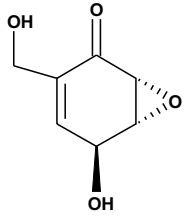
Arugosin A (66)



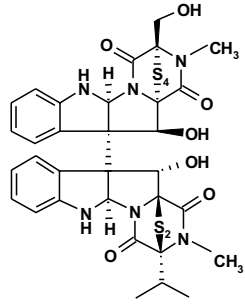
Arugosin B (67)



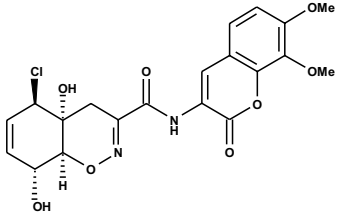
Chaetopyranin (68)



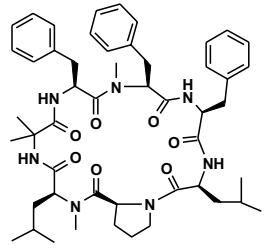
(+)-epiepoxydon (69)



Leptosin A (70)

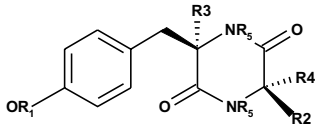


Trichodermamide B (71)

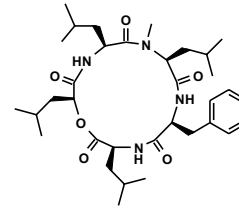


Scytalidamide A (72)

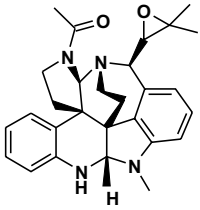
Fig. 5. Structures of cytotoxic metabolites isolated from endophytic fungi.



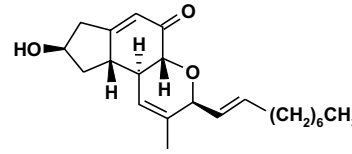
Fusaperzine A = R1=H, R2=H, R3=R4=SMe, R5=H (73)
Fusaperzine B = R1=isoprenyl, R2=SMe, R3=OMe, R4=R5=H (74)



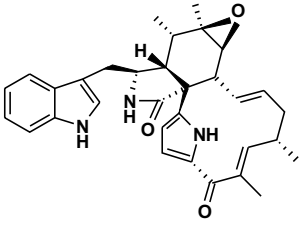
N-Methylsansalvamide (75)



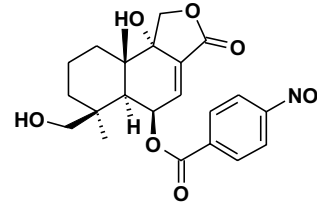
Communesins A (76)



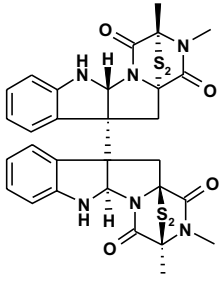
Penostatins A (77)



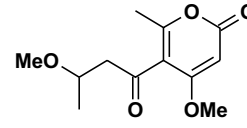
Penochalasins A (78)



Insulicolide A (79)



11,11'-Dideoxyverticillin A (80)



Pyrenocine E (81)

Fig. 5 continued. Structures of cytotoxic metabolites isolated from endophytic fungi.

intestinalis at Tanabe Bay is also a prolific fungus, and 19 new compounds, communesins A **(76)** (Fig. 5) and B, penostatins A **(77)** (Fig. 5) and B (–I), and penochalasins A **(78)** (Fig. 5), and B (–H) were obtained from this fungus (Numata et al., 1993, 1996; Takahashi et al., 1996; Iwamoto et al., 1998, 1999, 2001). These compounds exhibited potent cytotoxicity against P388 lymphocytic leukemia cell cultures with ED_{50} values of 0.3-0.9 µg/ml.

Aspergillus insulicola isolated from various algae in Bahamas (Rahbæk et al., 1997) and *Aspergillus versicolor* CNC 327 isolated from the green alga *Penicillus capitatus* also in Bahamas (Belofsky et al., 1998) gave insulicolide A **(79)** (Fig. 5), and three non-cytotoxic compounds. Insulicolide A exhibited a mean IC_{50} of 1.1 µg/ml in the NCI's 60-cell line panel.

Penicillium sp. CNC-350 isolated from the surface of the green alga *Avrainvillea longicaulis* in Bahamas gave two similar dimeric diketopiperazines, 11,11'-dideoxyverticillin A **(80)** (Fig. 5)**,** and 11'-deoxyverticillin A (Son et al., 1999), having potent cytotoxicity against the human colon carcinoma cell line HCT-116 (IC_{50} = 30 ng/ml).

Amagata et al. (1998) reported two compounds pyrenocines D and E **(81)** (Fig. 5)**,** which have been purified from *Penicillium waksmanii* isolated from the brown alga *Sargassum ringgoldianum*. Pyrenocine E showed cytotoxicity against P388 (ED_{50} = 1.30 µg/ml).

Outlook

Endophytic fungi are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants, marine algae) growing in so many unusual environments. Of the approximately 300,000 higher plant species that exist on the earth, each individual plant, of the millions that exist here, is host to one or more endophytes (Strobel et al., 2004). Similarly, numerous endophytic fungi are present in marine algae, residing inside the algal tissue. Mangroves are special woody plants community in the intertidal zone of the tropical and subtropical coast and harbour a large number of endophytic fungi. It seems obvious that endophytes are a rich and reliable source of genetic diversity. If endophytes can produce the same rare and important bioactive compounds as their host, this would not only reduce the need to harvest slow-growing and possibly rare plants/algae but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price.

The review of the literature indicates that novel chemotypes directed

against cancer have come from the microbial source, which is poorly investigated. Although the work on the utilization of endophytes has just begun, but it has already become obvious that they represent a promising source of novel metabolite.

As most of the plant diversity is found in tropical countries, there will be a great need of culture collection of this group of fungi with trained mycologists having classical and molecular background. The collection will help not only in getting novel secondary metabolites for various pharmaceutical and agricultural applications but also for applications like biotransformation, enzyme production etc.

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8 A Review of Antifungal and Antiviral Proteins

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Introduction

Antifungal and antiviral proteins have attracted the attention of many researchers owing to their economic implications. Fungal and viral infections may have devastating consequences on crops resulting in poor harvests and immense economic losses. Transgenic plants expressing antifungal proteins and antiviral proteins confer resistance to fungal and viral diseases (Shaw et al., 1994; Hong et al., 1996; Desmyter et al., 2003). Antifungal and antiviral proteins of diverse structures are produced by a variety of organisms including mammals, insects, gymnosperms, angiosperms, fungi and bacteria. Different plant tissues such as leaves, bark, roots, tubers, rhizomes and seeds may be the sources of these proteins (Harrison et al., 1991; Huynh et al., 1992a; Iijima et al., 1993). The objective of this chapter is to give an account of the diversity of antifungal and antiviral proteins that have been discovered to date. A look at Table 1 would give one the impression that antifungal and antiviral proteins with different N-terminal sequences can be isolated from different plant and mushroom species.

I. Plant Antifungal Proteins and Peptides

1. Allergen-like proteins

A 7.2-kDa peptide, resembling peanut allergen Ara H1 in N-terminal sequence, has been purified from seeds of the peanut *Arachis hypogea*. It

Table 1. N-terminal sequences of some antifungal and antiviral proteins

Rice bean defensin-like protein	RTHENLANTYKGPPITTG
Broad bean antifungal protein (fabin)	GDPGDQNGKA
Chestnut thaumatin-like protein	AKITFTNNHPRTIWP
Kiwi fruit thaumatin-like protein	ATFNFINNCPFTVWA
Green chickpea antifungal protein (cicerarin)	VKSTGRADDDLAVKTKYLPP
Pleurotus eryngii antifungal protein eryngin	ATRVVYCNRRSGSVVGGDDTVYYEG
Lyophyllum shimeijii antifungal protein	AGTEIVTCYNAGTKVPRGPSAXGGAIDFFN
Flammulina velutipes antiviral protein (velutin)	XHPDLFXXRPDNTASPKFEDPRLNP
Chinese ginseng antifungal/ antiviral protein (panaxagin)	GAHGARVYNIFRAALXRALN

exhibits antifungal activity against *Mycosphaerella arachidicola, Coprinus comatus* and *Fusarium oxysporum* (Ye and Ng, 2000b).

2. Arginine- and glutamate-rich proteins

Lilin is a novel 14.4-kDa arginine-and glutamate-rich protein with potent antifungal and mitogenic activities from bulbs of the lily, *Lilium browni*. It has the N-terminal sequence PRGRERYEYEAVRVRVQEAE. At a concentration of 120 μM, it almost completely abolishes the activity of HIV-1 reverse transcriptase (Wang and Ng, 2002a).

3. Chitinase-like proteins

A 28-kDa antifungal protein resembling chitinases in N-terminal sequence (Graham and Sticklen 1994) has been isolated from the field bean *Dolichos lablab*. The antifungal protein, designated as dolichin (Ye and NG, 2000a), and with the N-terminal sequence GAVGSVINASLFEQLLKHRND QDPEGKGFYSYNAFITA, inhibits mycelial growth in *Rhizoctonia solani* and *Fusarium oxysporum* at 300 μg per disk and *Coprinus comatus* at 60 μg per disk. In addition, it manifests some inhibitory activity toward HIV-1 reverse transcriptase, α-glucosidase and β-glucosidase (Ye and Ng, 2002a). HIV-1 reverse transcriptase is essential to the life cycle of the AIDS virus. The glucosidases are involved in viral coat glycosylation.

A 28-kDa chitinase-like protein has been purified from the seeds of

cowpea (*Vigna unguiculata*) (Ye et al., 2000c), pinto bean (*Phaseolus vulgaris* cv. 'Pinto Bean') (Ye and Ng, 2002c), and ricebean (*Delandia umbellata*) (Ye and Ng, 2002a). The chitinase-like protein from pinto beans augments macrophage production of nitric oxide (Ye and Ng, 2002c), while its counterpart from rice beans stimulates splenocyte proliferation (Ye et al., 2001b). These chitinase-like proteins exhibit a weaker antifungal action on *Rhizoctonia solani* than dolichin. They all demonstrate weak cell-free translating inhibiting activity and some HIV-1 reverse transcriptase inhibitory activity (Ye et al., 2000c; Ye and Ng, 2002c). The translation-inhibiting activity may account for, at least in part, the antifungal activity.

The pinto bean chitinase-like protein phasein A, with the N-terminal sequence CDVGSVISASLFEQQLKHRNGPAPARGDR, has a more potent antifungal activity toward *Fusarium oxysporum* than the 32-kDa novel pinto bean antifungal protein (Phasein B) with the N-terminal sequence GARKDDHAKLUFLLKDIEYO, and approximately equipotent to the 32-kDa protein toward *Physalospora piricola* (Ye and Ng, 2002c). Activity towards the various fungi are demonstrable at 60 μg per disk.

A 15-kDa antifungal protein, with an N-terminal sequence EQFGKQ AGMALQPNG similar to those of chitinases, has been isolated from *Panax notoginseng* (sanchi ginseng) roots. It demonstrates potent antifungal activity against *Fusarium oxysporum* at 20 μg (Lam and Ng, 2001a).

Two chitinases, chitinase A and chitinase B, both 28 kDa in molecular size, have been isolated from maize (*Zea mays*) seeds. They show 87% homology in amino acid sequence. Their N-amino acid sequences are MANAPRILAIGLLALLAAAGPAAAQN and QLVALGLALLCAVA GPAAAQN, respectively. Chitinase A is more active than chitinase B toward *Trichoderma reesei, Alternaria solani,* and *Fusarium oxysporum*. The binding constant of chitinase A against N, N′, N″, N‴-tetracetyl chitotetrose is considerably lower than that of chitinase B while chitinase A has a higher specific activity than chitinase B (Huynh et al. 1992a).

The chive (*Allium tuberosum*) chitinase-like protein, with an N-terminal sequence EQHGSQAGGALHPGXLHYSKYGGYGGTTPDYYGDGQQ showing a striking similarity to chitinases from leek (*Allium porrum*) and garlic (*A. sativum*), exerts antifungal activity against a variety of fungi such as *Rhizoctonia solani, Fusarium oxysporum, Mycosphaerella arachidicola* and *Botrytis cinerea*. The IC_{50} value of its antifungal activity against *B. cinerea* was 0.2 μM. Its antifungal activity is stable over a wide range of pH values (1.6 to 12.3) and at temperatures up to 60ºC for 5 minutes. It has mitogenic activity toward mouse splenocytes but exerts cytotoxicity on breast cancer cells (Lam et al., 2000).

4. Chitin-binding proteins

A 30-amino acid peptide with 6 Cys and 7 Gly residues, manifesting sequence similarity to the chitin-binding domain of chitin-binding chitinases and a more avid binding to chitin than chitin-binding chitinases, has been purified from intercellular washing fluid from sugar beet (*Beta vulgaris* L.) leaves. It exhibits antifungal activity against the devastating leaf pathogen *Cercospora beticola* (Sacc.) (Nielsen et al., 1997). The mRNA expression of this peptide is restricted to young flowers and leaves that are young and mature. However, no induction of expression of the peptide or its mRNA has been noted during infection with *C. beticola* or following treatment with dichloroisonicotinic acid, which is well-known to induce resistance in plants.

A 4244 Da chitin-binding protein, with an amino acid sequence and a cysteine/glycine rich chitin-binding domain typical of many chitin-binding proteins, has been isolated from *Ginkgo biloba* leaves. It exerts antifungal activity against *Alternaria alternata*, *Fusarium graminearum*, *F. moniliforme* and *Pellicularia sasakii* (Huang et al., 2000).

An ethylene-inducible chitin-binding protein, with antifungal activity toward several crop fungal pathogens, has been purified from guilder rose (*Hydrangea macrophylla*) leaves (Yang and Gong, 2002).

An antifungal protein, designated as CBP20, inducible by wound and tobacco mosaic virus, has been purified from tobacco (*Nicotiana tabacum* samsun NN) leaves (Ponstein et al., 1994). It lyses the germ tubes and/or slows the growth of *Trichoderma viride* and *Fusarium oxysporum*. It has an N-terminal chitin-binding domain and a C-terminal domain similar to tobacco and tomato pathogenesis-related proteins.

5. Cyclophilin-like proteins

A 18-kDa cyclophilin-like antifungal protein from black-eyed peas *Vigna unguiculata* L., with the N-terminal sequence FDMTAGPQPAGRIVFEGFAD MVGRTAVN, exhibits stronger antifungal activity toward *Mycosphaerella arachidicola* than *Botrytis cinerea* at 60 μg per disk (Ye and Ng et al., 2001a). Another cyclophilin-like antifungal protein from mungbean (*Vigna radiata*) seeds with a similar N-terminal sequence, designated as mungin, inhibits proliferation of mouse splenocytes. It does not inactivate HIV-1 reverse transcriptase although it attenuates the activities of α- and β-glucosidases. It inhibits the fungi *Rhizoctonia solani*, *Coprinus comatus*, *Mycosphaerella arachidicola*, *Botrytis cinerea* and *Fusarium oxysporum* at 60 μg per disk (Ye and Ng, 2000a).

6. Defensins

Peptides, from pinto beans (*P. vulgaris* L. c.v. 'Pinto Bean') and red beans (*P. angularis*), with a molecular weight of 5 kDa and N-terminal sequences KTCENLADTYKGPCET and RTCENLANTYRGPCI showing striking homology to those of cowpea (*V. unguiculata)* 10-kDa protein precursor and garden pea *(P. satirum)* disease response protein, potently inhibit mycelial growth in *Mycosphaerella arachidicola, Botrytis cinerea* and *Fusarium oxysporum* at 200 μg per disk. They also stimulate splenocyte proliferation (Ye and Ng, 2001b). A similar peptide with the N-terminal sequence RTHENLANTYKGPPITTG has been isolated from rice beans (Ye and Ng, 2002e).

Antifungal peptides from radish (*Raphanus sativus*) seeds with 50 or 51 amino acids belong to the plant defensin family. Linear synthetic 19-mer peptides have activity similar to the native antifungal proteins. Cysteines in the 19-mer peptides can be substituted by α-aminobutyric acid leading to an elevated antifungal activity. Analogous cyclic 19-mer peptides also demonstrate high antifungal activity (Schaaper et al., 2001).

A 37-residue antifungal protein, which possesses 6 Cys residues and the N-terminal sequence RPRPRPCIRAGGYCNILNVCLAGLTCEEH DIQDAACV, has been isolated from the intercellular washing fluid of sugar beet leaves. It suppresses growth of the fungal pathogen *Cercospora beticola* (Kristensen et al., 2001).

The cDNA cloning, expression in *Pichia pastoris,* isolation, and characterization of recombinant *Pisum sativum* defensin 1, a Cys-rich antifungal protein with four disulfide bridges, have been reported (Almeida et al., 2001).

7. Deoxyribonucleases

A 30-kDa deoxyribonuclease, with a novel N-terminal sequence GIEVIKIREL and antifungal activity against *Botrytis cinerea* at 150 μg per disk but not against *Fusarium oxysporum, Mycosphaerella arachidicola* and *Rhizoctonia solani,* has been isolated from asparagus seeds (*Asparagus officinalis*). A pH of 7.5 is required for optimal DNase activity toward herring sperm DNA. It inhibits cell-free translation with an IC_{50} of 20 μM but does not affect HIV-1 reverse transcriptase (Wang and Ng, 2001b).

8. Embryo-abundant proteins

Ginkbilobin, a 13-kDa antifungal protein from *Ginkgo biloba* seeds, displays an N-terminal sequence ANTAFVSSAHNTQKZPAGAPFNRNLRAMLA DLRGQNAAFAG analogous to embryo-abundant protein from the white

spruce. It potently inhibits fungal growth in *Botrytis cinerea, Mycosphaerella arachidicola, Fusarium oxysporum*, *Coprinus comatus,* and *Rhizoctonia solani* with an IC_{50} of 0.25 μM, 6.5 μM, 3.6 μM, 3.4 μM and 8.7 μM, respectively. It also exerts moderate antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. It attenuates the mitogenic response in mouse splenocytes and inhibits HIV-1 reverse transcriptase (Wang and Ng, 2000b).

9. Glucanases

A glucanase and two chitinases have been prepared from chickpea (*Cicer arietinum*) (Vogelsang and Barz, 1993). Novel antifungal peptides designated cicerin and arietin have been isolated from chickpea seeds (Ye et al., 2002). An antifungal peptide named cicerarin with the N-terminal sequence VKSTGRADDDLAVKTKYLPP has been purified from green chickpea seeds (Chu et al., 2003).

10. Hemagglutinins/lectins

A homodimeric 67-kDa hemagglutinin from red kidney bean (*Phaseolus vulgaris*) seeds, with the N-terminal sequence ANQTSFNFQRFDCETNLIL QRDATVSSKGQLRLTNVNDNPEEPTVDNLGRA, demonstrates moderate antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani* at 60 μg per disk (Ye et al., 2001a). Antifungal activity in stinging nettle lectin (Broekaert et al., 1989) and potato tuber lectin (Gozia et al., 1995) has previously been reported.

11. Lipid transfer proteins

A 9.4-kDa peptide with the N-terminal sequence ALSCGTVSGNLA ACAGYV was isolated from seeds of *Brassica campestris* L. var. *purpurea Bailey*. The IC_{50} of its antifungal activity against *M. arachidicola* and *F. oxysporum* were 4.5 μM and 8.3 μM, respectively. A 10-kDa protein, designated as Ace-AMP1, with antifungal and antibacterial activity toward Gram-positive bacteria, and sequence resemblance to plant lipid transfer proteins, has been purified from onion seeds. Its N-terminal sequence is QMICPRVNRIVIPCVAYGL. The protein is incapable of effecting phospholipid transfer from liposomes to mitochondria, unlike radish and maize nonspecific lipid transfer proteins. It has only 76% of the conserved amino acid residues in known plant nonspecific lipid transfer proteins. Its antifungal activity is hardly affected by the presence of cations at physiological concentrations. On the contrary, the antifungal activity of radish lipid transfer protein is observable only at low cation concentrations

(Terras et al., 1992). Maize and wheat lipid transfer proteins have little or no antimicrobial activity (Cammue et al., 1995). The solution structure of Ace-AMP1 has been elucidated (Tassin et al., 1998).

12. Miraculin-like proteins

A 38-kDa antifungal protein, with an N-terminal sequence APNVAPDV GHADLRA similar to miraculin and antifungal activity toward *Fusarium oxysporum*, has been isolated from sugar snap (*Pisum sativum* var. *macrocarpon*) legumes (Ye et al., 2000b).

13. Peroxidase

A 37-kDa peroxidase, with antifungal activity against *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola* and an N-terminal sequence LDNPAGFKGDKANGMLKMGNLCFLAKA, has been purified from French bean (*Phaseolus vulgaris* cv. 'French Bean') legumes (Ye and Ng, 2002d). It has been shown that pathogen induces peroxidase production in barley coleoptile (Kristensen et al., 1999).

14. Protease inhibitors

A Bowman-Birk type trypsin-chymotrypsin inhibitor from broad bean (*Vicia faba*) seeds, with the N-terminal sequence GDKVKSACCDTTLLKKKEH PPPRR suppresses mycelial growth in *Mycosphaerella arachidicola*, *Fusarium oxysporum* and *Botrytis cinerea* (Ye et al., 2001b). The IC_{50} value of its antifungal activity toward *B. cinerea* was 51 μM. It inhibits HIV-1 reverse transcriptase with an IC_{50} of 40 μM and demonstrates mitogenic activity toward murine splenocytes.

A 24-kDa cystine protease inhibitor from pearl millet (*Pennisetum glaucum*) exhibits potent antifungal activity against *Trichoderma reesei*, and *Claviceps*, *Helminthosporium*, *Curvularia*, *Alternaria* and *Fusarium* species (Joshi et al., 1998).

15. Ribonucleases

From Chinese ginseng (*Panax ginseng*) roots an antifungal protein with ribonuclease activity has been isolated. The protein, designated as panaxagin, has two identical 26-kDa subunits with the N-terminal sequence GAHGARPYNIFRAALXRALN. Its HIV-1 reverse transcriptase inhibiting activity can be heightened by succinylation using succinic anhydride (Ng and Wang, 2001). A heterodimeric ribonuclease with

antifungal activity has been isolated from sanchi ginseng roots (Lam and Ng, 2001b).

American ginseng (*Panax quinquefolium*) antifungal protein, with the N-terminal sequence GAHGARVYNIDRNDV, is a homodimeric 53-kDa protein with specific ribonucleolytic activity toward poly C. Its HIV-1 reverse transcriptase inhibitory activity can also be enhanced by sucinylation. The protein, designated as quinqueginsin, inhibits cell-free translation with an IC_{50} of 0.26 nM (Wang and Ng, 2000a).

16. Ribosome inactivating peptides

Luffacylin, an arginine-and glutamate-rich ribosome-inactivating peptide with antifungal activity and the N-terminal sequence PRGSPRTEYEAARR, has been isolated from *Luffa cylindrica* seeds (Parkash et al., 2002).

17. Ribosome inactivating proteins

From dehusked barley (*Hordeum vulgare*) grains a 30-kDa ribosome-inactivating protein with antifungal activity, and a 28-kDa antifungal protein with a 20-fold more potent antifungal activity, have been isolated (Roberts and Selitrennikoff, 1986). The latter suppresses mycelial growth in *Trichoderma reesei, Phycomyces blakesleeanus, Alternaria alternaria* and a protoplast-forming mutant of *Neurospora crassa* (Roberts and Selitrennikoff, 1986; Leah et al., 1991).

Wheat (*Triticum vulgaris*) pathogenesis-related antifungal proteins, designated as wheatwin 1, wheatwin 2, wheatwin 3 and wheatwin 4, have been reported. Wheatwin 3 differs from wheatwin 1 by one amino acid at position 88. Wheatwin 4 differs from wheatwin 2 by one amino acid at position 78 (Caruso et al., 1996, 2001).

18. Thaumatin-like proteins

A 20-kDa thaumatin-like antifungal protein capable of inhibiting mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola* with an IC_{50} of 20 μM for the former has been isolated from the emperor banana *Musa basjoo*. It possesses the N-terminal sequence ATAFFEFVNRCCYTVAAAAV (Ho et al., 2007).

A 20-kDa thaumatin-like antifungal protein, with an N-terminal sequence ANFEIVNNCPYTVWAAASPGGGRR-LDRGQT, has been prepared from French bean (*Phaseolus vulgaris* cv. 'Kentucky Wonder') legumes (Ye et al., 1999). It is active against *Coprinus comatus* at 60 μg per disk, but only weakly active against *Rhizoctonia solani* and *Fusarium*

oxysporum. Regalado and Ricardo (1996) purified and characterized a thaumatin-like protein and a chitinase from intercellular fluid of *Lupinus albus* leaf, stem and root tissues. Thaumatin-like proteins, induced by infection with *Ascochyta rabiei*, have been isolated from intercellular washing fluid of chickpea (*Cicer arietinum* L.) leaves (Hanselle et al., 2001).

A 27-kDa thaumatin-like antifungal protein has been reported from *Diospyros texana* fruits (Vu and Huynh, 1994). A 21-kDa thaumatin-like antifungal protein from kiwi fruits, with an N-terminal sequence ATFNFINNCPFTVWAAAVPG and inhibitory activity on *Botrytis cinerea* and HIV-1 reverse transcriptase (Wang and Ng, 2002b), has been isolated. The IC_{50} values of its antifungal activity against *B. cinereus* and *M. arachidicola* were respectively 0.43 μM and 8 μM.

Barley (*Hordeum vulgare*) thaumatin-like proteins exert antifungal activity against *Trichoderma viride* and *Candida albicans*. The activity is synergistic with that of nikkomycin Z, a nucleoside-peptide inhibitor of fungal chitin synthesis (Hejgaard et al., 1991).

A thaumatin-like protein, a β-1,3-glucanase precursor, and an antifungal protein, are co-induced by the signaling compound jasmonic acid in rice (*Oryza sativa*) leaf tissues (Rakwal et al., 1999). A 22-kDa thaumatin-like protein has been purified from maize seeds (Huynh et al., 1992a,b).

The mRNA level of thaumatin-like protein is maximal in the ripe cherry (*Prunus avium* L.) fruits. However, antifungal activity of the protein is not observed (Fils-Lycaon et al., 1996).

The 25-kDa flax seed antifungal protein suppresses the growth of *Alternaria solani* and synergizes the antifungal action of nikkomycin Z toward *Candida albicans*. It shows marked sequence homology to other pathogenesis-related antifungal proteins (Bergmeyer et al., 1992).

A 30-kDa thaumatin-like antifungal protein with the N-terminal sequence AKITFTNNHPRTIWP has been purified from chestnut (*Castanopsis chinensis*) seeds (Chu and Ng, 2003a). It exerts antifungal activity against *Fusarium oxysporm* with an IC_{50} of 0.5 μM. Another thaumatin-like protein was isolated from seeds of the chestnut *Castanea mollisima* (Chu and Ng, 2003b). It exerts antifungal activity with an IC_{50} of 0.83 μM against *Fusarium oxysporum*, 6.48 μM against *Mycosphaerella arachidicola* and 9.21 μM against *Physalospora piricola*.

Dendrocin is an antifungal protein from bamboo shoots with a N-terminal sequence TTLTLHNLCPYPVWWLVTPNNGGYPIIDNTPVVLG similar to thaumatin-like proteins (Wang and Ng, 2003a). The IC_{50} values of its antifungal activity toward *B. cinera*, *F. oxysporum* and *M. arachidicola* were, respectively, 1.8, 1.4 and 5.1 μM.

19. γ-thionins

Barley and wheat α-thionins are antimicrobial peptides that are able to bind to polysaccharide containing chitin and β-1,3-glucan which compose the fungal cell wall (Oita et al., 2000).

Three antifungal γ-thionins from yellow mustard (*Sinapsis alba* L.) seeds are substrates for plant calcium-dependent protein kinase (Neumann et al., 1996).

A gamma-thionin protein SIα1 from *Sorghum bicolor* seeds with structural similarity to scorpion proteins demonstrates antifungal activity (Bloch et al., 1998).

20. Cysteine-rich proteins

Highly basic hevein-like peptides from *Pharbitis nil* seeds possess characteristics of a cysteine/glycine-rich domain (Koo et al., 1998).

Four macrocyclic cystine-knot peptides with 29-31 residues from coffee plants exert moderate activity against *Candida kefyr* and *C. tropicalis* (Tam et al., 1999).

A cysteine-rich protein capable of inhibiting the growth of filamentous fungi and gram-positive bacteria, has been isolated from seeds of the pokeweed (*Phytolacca americana*). It shows sequence homology to *Mirabilis jalapa* antimicrobial protein (Liu et al., 2000).

A basic 3929-Da peptide from pokeweed seeds, with a broad spectrum of antifungal activity, displays substantial sequence homology and the same cysteine motif with knottin-type antimicrobial peptides from *Mirabilis jalapa* seeds (Shao et al., 1999).

21. Novel proteins

Chrysancorin from garland chrysanthemum seeds has an N-terminus that manifests sequence homology to the genomic sequence of chromosome 1 in *Arabidopsis thaliana* BAC T19E23 (Walker et al., 1999). It evokes a mitogenic response from murine splenocytes and exhibits HIV-1 reverse transcriptase inhibitory activity. Antifungal activity toward *Botrytis cinerea, Physalospora piricola* and *Mycosphaerella arachidicola* is observed. However, no antibacterial activity is noted (Wang and Ng, 2001a).

An antifungal protein, with a novel N-terminal sequence DTFSDA GSFLDRGLAKSDDDAARRQFQPNNRYFTGGNKGAQGVVDGHHGL and designated as allivin, has been isolated from bulbs of the single-cloved garlic (Wang and Ng, 2001c). The protein is active against *M. arachidicola* at 6 μg per disk and against *B. cinerea* and *P. piricola* at 30 μg per disk.

From the pinto bean a novel 32-kDa antifungal protein with pronounced macrophage-stimulating activity has been reported (Ye and Ng, 2002c). An antifungal peptide designated as angularin, 8 kDa in molecular weight, and exhibiting antifungal activity against *Mycosphaerella arachidicola* and *Botrytis cinerea,* has been isolated from red beans (Ye and Ng, 2002b). Two 4 to 5 kDa antifungal peptides from Ceylon spinach (*Basella rubra*) seeds produce an antifungal action against *Botrytis cinerea, Mycosphaerella arachidicola* and *Fusarium oxysporum* (Wang and Ng, 2001d). β-basrubin has the N-terminal sequence KIMAKPSKFYBQLRGR and α-basrubin has the N-terminal sequence GADFQECMKEHSQKQHQHQ. Both antifungal peptides are active against *B. cinerea*, *F. oxysporum* and *M. arachidicola* at 3.2 μg per disk. There is no activity toward *C. comatus* and *R. Solani*.

Snakin-1, an antimicrobial peptide from potato tubers, synergizes with potato defensin PTH1 in antifungal action against *Botrytis cinerea* and antibacterial action against *Clavibacter michiganensis sub sp. sepedonicus.* Its pattern of gene expression suggests that it is a component of constitutive defense barriers, especially those of storage and reproductive organs (Segura et al., 1999).

Four highly basic 20-amino acid peptides with 2 disulfide bonds from *Impatiens balsamina* seeds have a growth-retarding effect on various fungi and bacteria. The predicted precursor of these antimicrobial peptides has a novel structure composed of repeated alternating basic mature peptide domains and acidic propeptide domains (Tailor et al., 1997).

Alocasin is a 11-kDa protein from giant taro *(Alocasia macrorhiza)* rhizomes. It inhibits only *B. cinerea* out of the several fungi tested (Wang and Ng, 2003b).

The black soybean produces a 25-kDa antifungal protein with antimitogenic and HIV-1 reverse transcriptase inhibiting activities (Ngai and Ng, 2003a). Fabin from the broad bean possesses an N-terminal sequence GDPGDQNGKA similar to glucanase and calcyon (Ng and Ye, 2003). It demonstrates, mitogenic and translation-inhibiting activities, and also antifungal activity against *B. cinerea*, *F. oxysporum*, *M. arachidicola* and *R. solani* at 60 μg per disk.

Two antimicrobial peptides from *Mirabilis jalapa* seeds, designated as Mj-AMP1 and Mj-AMP2, are closely related in sequence and exhibit activity against gram-positive bacteria and a large number of fungi. Mj-AMP2, being more potent in antifungal activity, has an IC_{50} of 0.5-20 μg/ml compared with the value of 6-30 μg/ml for Mj-AMP1. They show sequence homology to mu-agatoxin, a class of insecticidal neurotoxic peptides from spider venom (Cammue et al., 1992).

The *in vitro* antifungal activity of *Mirabilis jalapa* and *Amaranthus*

caudatus antimicrobial peptides, purified from transgenic tobacco plants expressing their precursor proteins, is about the same as that of the authentic proteins. Nevertheless, the transgenic plants do not acquire a higher resistance against infection with either *Botrytis cinerea* or *Alternaria longpipes* (De Bolle et al., 1996).

Structure-function relationship in the case of *Amaranthus candatus* antimicrobial peptide 2 has been examined. The replacement of any aromatic acid by alanine and the mutation of any aromatic acid to alanine, and the mutation of tyrosine 20 to tryptophan, ensue in a decrease in chitin affinity. The mutation of phenylanine 18 to a larger aromatic residue increases the affinity for chitin (Muraki et al., 2000).

A 4-kDa basic peptide without sequence similarity to thionins has been purified from maize (inbred B73) kernels. It retards spore germination and hyphal elongation in the maize pathogens *F. moniliforme* and *F. graminearum*, a bacterial pathogen of maize, and several bacteria (Duvick et al., 1992).

During seed germination in cheeseweed *(Malva parviflora)*, cigar tree *(Catalpa speciosa)* and wheat, a larger number of antifungal proteins appear, and the antifungal activity shifts from small molecules to larger molecules. Random proteolytic digestion of seed protein does not generate antifungal proteins. This increased production of antifungal protein may play a critical role in plant protection during early developmental stages (Wang et al., 2002).

A subtractive hybridization technique has been employed to clone cDNAs representing genes that show increased expression during leaf senescence in *Brassica napus.* One of the genes products is an antifungal protein (Buchanan-Wollaston and Ainsworth, 1997).

The three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 (Rs-AFP1) has been investigated with the use of ^{1}H NMR. The molecule adopts a compact globular fold comprised of an α-helix and a triple-stranded β-sheet with the middle strand connected by two disulfide bridges to the α-helix. A type via β-turn links β-strands 2 and 3. The loop between β-strand 1 and the α-helix is relatively well-defined. The structural features have all the characteristics of the cysteine-stabilized α-, β motif (Fant et al., 1998).

The crystal structure of zeamatin, antifungal protein from *Zea mays,* has been reported (Batalia et al., 1996). Like maize zeamatin, protein R and S from barley grains inhibit fungal growth in synergism with nikkomycin Z, a nucleoside-peptide inhibitor of fungal chitin synthesis (Hejgaard, 1991).

II. Antifungal Proteins from Mushrooms

A 14-kDa antifungal protein, with an N-terminal sequence AGTEIVTCYNA GTKUPRGPSA-XGGAIDFFN, which exhibits slight resemblance to angiosperm thaumatin-like proteins, has been purified from fruiting bodies of the mushroom *Lyophyllum shimeiji*. Its antifungal activity is more potent than the ribosome-inactivating protein isolated from the same mushroom. Both proteins potently inhibit HIV-1 reverse transcriptase, with IC_{50} values approximating 5 nM and 8 nM, respectively (Lam and Ng, 2001c). Antifungal proteins have also been purified from *Lentinus edodes, Pleurotus alveolaris* and *Pleurotus eryngii* (Ngai and Ng, 2003a; Wang and Ng, 2004; Wang et al., 2004). Their N-terminal sequences were, respectively, CQRAFNNPRDDAIRW, GVCDMADLA, and ATRVVYLNRRSGSVVG GDDTVYYEG. The antifungal protein from *L. edodes* exerts antifungal activity against *M. arachidicola* with an IC_{50} of 17.5 μM. The IC_{50} values of *P. eryngii* antifungal protein against *M. arachidicole* and *F. oxysporum* were 3.5 μM and 1.35 μM.

Lentinus edodes and *Irpex lacteus* produce thaumatin-like proteins (Grenier et al., 2000).

III. Antifungal Proteins from Other Fungi

The antifungal peptide from *Aspergillus giganteus* inhibits the growth of many filamentous fungi but not yeasts or bacteria. It does not inhibit the growth of *Aspergillus giganteus.* It requires a minimum concentration of 6-25 μM of the antifungal peptide to totally inhibit fungal growth. It is recalcitrant to proteolysis and does not show any thermal transition up to 80°C as revealed by differential scanning calorimetry and infrared spectroscopy. It demonstrates a strong positive band at 230 nm as a conspicuous feature of the circular dichroism spectrum in the far ultraviolet region. It is rich in tyrosine residues which can be divided into an exposed population and a buried population based on data from pH-titration experiments. A high content of β-structure is disclosed by Fourier-transform infrared spectroscopy. A rather unstructured polypeptide is formed as a result of reduction and carboxyamidomethylation according to spectroscopic results (Lacadena et al., 1992).

The antifungal protein from the fungus *Aspergillus giganteus* has been sequenced (Nakaya et al., 1990). It demonstrates strong antifungal activity against the phytopathogenic fungi *Magnaporthe grisea* and *Fusarium moniliforme* and the oomycete pathogen *Phytophthora infestans* (Vila et al., 2001). Heterologous expression of the gene encoding this antifungal protein reduces the formation of powdery mildew (*Erysiphe graminis* f. sp. *tritici*) or leaf rust (*Puccinia recondite* f. sp. *tritici*) in transgenic wheat compared

with non-transgenic controls (Oldach et al., 2001).

The solution structure of *Aspergillus giganteus* antifungal protein has been determined. Its folding comprises five antiparallel β-strands connected in –1, –1, +3, +1, topology and highly twisted, defining a small and compact β barrel stabilized by four internal disulfide bridges. A cationic site constituted by up to three lysine side chains next to a hydrophobic stretch, both at the protein surface, may form a potential phospholipid binding site which would furnish the basis of its antifungal activity (Campos-Olivas et al., 1995).

A 12-kDa protein, with sequence homology to *Aspergillus giganteus* and antifungal activity, has been purified from the culture medium of *Penicillium chrysogenum* (Marx et al., 1995).

An antifungal peptide, with 58 amino acids and 6 Cys residues, has been isolated from the culture medium of *Aspergillus niger.* It exhibits a certain extent of sequence homology to cysteine-rich antifungal peptides reported from the seeds of *Sinapsis alba* and *Arabidopsis thaliana* or the extracellular media of *Aspergillus giganteus* and *Penicillium chrysogenumsome*. Potent inhibitory activity against yeast and filamentous algae has been observed (Gun Lee et al., 1999).

Cryptocandin is a lipopeptide with a molecular weight of about 1.1 kDa from the endophytic fungus *Cryptosporiopsis* cf. *quercina*, chemically related to fungal antimycotics including echinocandins and pneumocandins. Its structure is glutamine (1)-3-hydroxy-4-hydroxy methylproline(2)-4,5-dihydroxyornithine(3)-palmitic acid-threonine(4)-4-hydroxy-proline(5)-3,4-dihydroxy-homotyrosine(6) where the first amino acid (1) is joined to the 6th amino acid (6) to form a cyclic structure. It is inhibitory toward *Candida albicans, Trichophyton mentagrophytes, T. rubrum, Sclerotina sclerotiorum* and *Botrytis cinerea* (Strobel et al., 1999).

Viridin is a 65-kDa antifungal protein isolated from the culture medium of the mold *Trichoderma viride* (Hao et al., 1999). The same group of investigators have isolated an antifungal protein in addition to α-sarcin from *Aspergillus giganteus* (Hao et al., 1998). The antifungal protein inhibits hyphal growth in *Verticillium dahliae*.

The cDNA encoding the antifungal protein KP4 from *Ustilago maydis* infecting virus has been inserted behind the maize ubiquitin promoter and transferred to varieties of wheat that are susceptible to stinking smut (*Tilletia tritici*) disease. KP4-transgenic wheat plants have a higher resistance against stinking smut (Clausen et al., 2000).

Cloning, sequence analysis and expression of the gene encoding antifungal proteins from *Aspergillus giganteus* and *Raphanus sativus* have been achieved (Alves et al., 1994; Wnendt et al., 1994).

IV. Antifungal Peptides from Bacteria

Antifungal peptides with inhibitory activity toward *Alternaria brassiciola* have been demonstrated in the fermentation broth of *Bacillus subtilis* CL2T (Leifert et al., 1995).

Iturin A and surfactin are antifungal lipopeptides from *Bacillus subtilis*. The purification protocol comprises solid-phase extraction on C18 gel and reversed-phase chromatography employing a biocompatible Pep RPC HR 5/5 column with an FPLC system (Razafindralambo et al., 1993).

A lipopeptide, AFC-BC11, largely associated with the cell membrane, has been characterized from a soil isolate of *Burkholderia cepacia* BC11. It accounts for the ability of BC11 to effectively control the damping-off of cotton caused by the fungal pathogen *Rhizoctonia solani* (Kang et al., 1998).

Tensin is an antifungal cyclic lipopeptide from *Pseudomonas fluorescens*. It causes a reduction in radial mycelial extension but an increase in branching and resette formation in *Rhizoctonia solani* (Nielsen et al., 2000).

Viscosinamiole is a new cyclic depsipeptide with surfactant and antifungal activities from *Pseudomonas fluorescens*. Its structure is β-hydroxy-decanoyl-L-leucine(1)-D-glutamine(2)-D-allo-threonine(3)-D-valine(4)-L-leucine(5)-D-serine(6)-L-leucine(7)-D-serine(8)-L-isoleucine(9) in which amino acid residue no. 3 is joined to amino acid residue no. 9 to form a cyclic structure. It shows inhibitory activity toward the phytopathogenic fungi *Pythium ultimum* and *Rhizoctonia solani in vitro* as well as *in planta* (Nielsen et al., 1999).

Pseudomycin A from liquid culture of *Pseudomonas syringae* is a 1.2 kDa peptide made up of hydroxyaspartic acid, aspartic acid, diamino-butyric acid, lysine, arginine and serine. Prominent activity against the human pathogen *Candida albicans* is observed (Harrison et al., 1991).

Ecomycins are antimycotic peptides with a molecular weight of about 1.1 kDa, isolated from *Pseudomonas viridiflava*, a plant-associated bacterium. They show activity against a wide range of phytopathogenic fungi (Miller et al., 1998).

A chitin-binding protein with antifungal activity has been purified from the culture filtrate of *Streptomyces tendae* Tu 901. It exhibits a molecular weight of 9.86 kDa and synergistic interaction with the chitin synthetase inhibitor nikkomycin on growth of *Aspergillus species*. The chitin-binding protein demonstrates strong binding to the surface of germinating conidia and to tips of growing hyphae, resulting in large spherical conidia, swollen hyphae and atypical branching (Bormann et al., 1999).

V. Antifungal Proteins from Insects

The full sequence of cecropin A is MNFVRILSFVFALVLALGAVSAA PEPRWKLFKKIEKVGRNVRDGLIKAGPAIAVIGGAKSLGK Cecropin A—derived peptides, smaller than cecropin A which is a naturally occurring peptide with a significant role in the insect immune response, have been synthesized by Cavallavin et al. (1998) and tested for antifungal activity. An 11-amio peptide corresponding to the N-terminal amphipathic α-helix domain of cecropin A demonstrates strong antifungal activity against *Phytophthora infestans* (IC_{50} = 2 μM).

Antifungal proteins have also been isolated from insects. Cicadin is a 6.5-kDa antifungal peptide from dried juvenile cicadas with the N-terminal sequence NEYHGFVDKANNENKRKKQQGRDDFVVKPNNFANRRRK DDYNENYYDDVDAADVV. It exerts potent antifungal activity with IC_{50} values at nanomolar concentrations (Wang and Ng, 2002c). The purification and cDNA cloning of an antifungal protein from the hemolymph of *Holotrichia diomphalia* larvae have been achieved (Lee et al., 1995).

Drosomycin, induced by septic injury of the fruitfly *Drosophila melanogaster* (Fehlbaum et al., 1994), is a 44-residue antifungal peptide. Its solution structure involves a three-stranded β-sheet and an α-helix, with four disulfide bridges maintaining the protein global fold. It shows 41% sequence similarity with *Raphanus sativus* antifungal protein 2 (Rs-AFP2). Structural analysis of drosomycin and Rs-AFP2 disclosed the sharing by the two antifungal proteins of a hydrophobic cluster located at the protein surface in which a lysine residue is embedded. The active site of drosomycin has been proposed based on the structural similarity between the two antifungal proteins and the results regarding the antifungal activity of Rs-AFP2 mutants (Landon et al., 1997, 2000).

A synthetic cDNA of *Drosophila melanogaster* has been expressed in *Saccharomyces cerevisiae,* using the mating factor alpha gene and simultaneously overexpressed KEX2 gene to increase the yield of fully expressed drosomycin. Drosomycin has been shown by Edman degradation and mass spectrometry to have sequence similarity to plant defensins and the same array of intramolecular S-S bonds (Michaut et al., 1996).

Heliomicin from the lepidopteran *Heliothis virescens* possesses both antifungal and antibacterial activities (Lamberty et al., 2001).

An antifungal protein has been purified from the hemolymph of third instar larvae of *Sarcophaga peregrina* (Iijima et al., 1993). It inhibits the growth of *Candida albicans*.

Tenecin 3 is an antifungal protein from the insect *Tenebrio molitor* which

inhibits the growth of *Candida albicans*. Its full sequence is MKTFVICLI LVVAVSAAPDHHDGHLGGHQTGHGGGGGGGH2GGGGGGHLGG HGGGGPGGHLGGHGGGIGGTGGQQHGQHGPGTQAGHQGGYKTHGH. Its structural characteristics have been studied. It has a random structural propensity that is not significantly affected by temperature, pH, organic solvents and sodium dodecyl sulfate micelles (Lee et al., 1999a). It acquires higher antifungal activity after conjugation with polyethylene glycol but it does not interact with mammalian cells (Lee et al., 1996, 1999a, 1999b).

VI. Antifungal Peptides from Mammals

Hepecidin 20 and hepecidin 25 are two cysteine-rich peptides originating from the liver and found in the urine. They manifest antifungal activity against *Aspergillus fumigatus*, *A. niger* and *Candida albicans* as well as bacteria (Park et al., 2001).

Mechanisms of action of some antifungal proteins

Investigations on the mechanism of antifungal action for some of the aforementioned proteins and peptides have been carried out. Thaumatin-like proteins may destabilize fungal membranes and bind and/or hydrolyze fungal β-1,3-glucans. Some thaumatin-like proteins like tobacco osmotin stimulate a mitogen-activated protein kinase signal translation mechanism to induce changes in the fungal wall that increase toxicity (Yun et al., 1998; Grenier et al., 1999). Chitinases may hydrolyze chitin, a polymer of N-acetylglucosamine and an important component of the fungal wall (Graham and Sticklen, 1994). However, the mechanisms of action of many other antifungal proteins and peptides await clarification.

VII. Plant Antiviral Proteins

Antiviral activity has been detected in the extracts of many plants (Chessin, 1995; Verma et al., 1995a,b). In some cases the antiviral principle has been isolated and chemically identified (Bonness et al., 1994; Verma et al., 1995a,b). In addition to alkaloids, flavonoids, furocoumarins, hypericins, lignans, tannins, polysaccharides, terpenoids and thiophenes (Hudson, 1995; Ng et al., 1997), plants produce antiviral proteins (Bol et al., 1995; Gera et al., 1995; Mitra, 1995). Ribosome inactivating proteins (RIPs) represent the best-studied group of plant antiviral proteins. The list of RIPs that have been purified and characterized has been expanding (Battelli and Stirpe, 1995; Irwin, 1995; Zipf, 1995). Type 1 RIPs, which are single-chained proteins with a molecular mass close to 30 kDa, have been isolated from the following families: Asparagaceae, Caryophyllaceae, Cucur-

bitaceae, Euphorbiaceae, Nyctaginaceae, Phytotaccaceae and Poaceae (Ng et al., 1992; Shaw et al., 1994; Battelli and Stirpe, 1995) Type 1 RIPs have been shown to inhibit infection caused by plant viruses including African cassava mosaic virus, alfafa mosaic virus, cucumber mosaic virus, potato virus X, potato virus Y, southern bean mosaic virus, tobacco mosaic virus, and tobacco necrosis virus. Animal viruses including herpes simplex virus-1 influenza virus and poliovirus are inhibited by type 1 RIPs. Some type 1 RIPs are capable of inhibiting HIV-1 replication and the activities of the enzymes crucial to the life cycle of the HIV-1 including reverse transcriptase, protease and integrase (Au et al., 2000). Type 2 RIPs, composed of an RIP chain and a lectin chain, have been purified from members of the Euphorbiaceae, Fabaceae, Passifloraceae, Sambucaceae and Viscaceae families (Battelli and Stirpe, 1995; Chen et al., 2002). Type 2 RIPs inhibit tobacco mosaic virus (Battelli and Stirpe, 1995).

The *in planta* antiviral activity of the type 2 RIPs from *Sambucus nigra* (Chen et al., 2002) is attributed to their polynucleoticle-adenosine glycosylase activity toward tobacco mosaic virus RNA (Vandenbussche et al., 2004).

Expression of a *Phytolacca insularis* antiviral protein (ribosome inactivating protein) gene is increased considerably in leaves after mechanical wounding and treatment with jasmonic acid and abscisic acid (Song et al., 2000). RIP is also induced by stress in the common ice plant *Mesembryanthemum crystallinum* (Rippmann et al., 1997).

RIPs are characterized by the ability to inhibit protein synthesis by rabbit reticulocyte lysate. They exhibit RNA N-glycosidase activity and consequently depurinate, RNA (Bolognesi et al., 1990, 1997; Taylor and Irvin, 1990; Barbieri et al., 1992; Kataoka et al., 1992; Parente et al., 1993; Olivieri et al., 1996; Desmyter et al., 2003). The DNase (Day et al., 1998) and RNase (Wang et al., 2003) activities of RIPs have been shown to be due to contaminants. Polynucleotide: adenosine glycosidase is the sole activity of RIPs on DNA (Barbieri et al., 2000).

Expression of RIP gene has been found in abundance in leaves of *Dianthus sinensis* (Cho et al., 2000). RIPs have been isolated from different tissues of the same plant (Battelli and Stirpe, 1995). Different forms of RIP may exist in the same plant tissue e.g. *Phytolacca americana* seeds (Desvoyes et al., 1995; Honjo et al., 2002; Vivanco et al., 1999).

Native polleweed (*Phytolacca americana*) antiviral protein has been used clinically as the active moiety of immunoconjugate against cancer and AIDS. Large-scale expression and purification of biologically active recombinant pokeweed antiviral protein was first achieved by Rajamohan et al. (1999). It has been demonstrated that an intact active site, as well as an intact C-terminal, are required for toxicity of pokeweed antiviral protein

and its depurination of tobacco ribosomes *in vivo*. However, an intact active site, but not an intact C-terminus, is crucial to the antiviral activity, indicating a dissociation of antiviral activity from toxicity (Tumer et al., 1997).

Resistance to a plant DNA virus, African cassava mosaic virus, had been achieved by virus-induced expression of the RIP dianthin in transgenic *Nisotiana denthamiana* plants (Hong et al., 1996).

Monoclonal antibodies against pokeweed antiviral protein have been raised. They represent a useful tool for studying the mechanisms of RIP biosynthesis and plant protection involving RIPs (Desvoyes et al., 1995). Some lectins manifest inhibitory activity against viruses including HIV-1 and bovine immunodeficiency virus and HIV-1 reverse transcriptase (Ng et al., 1997; Ooi et al., 2000; Wang and Ng, 2001e).

VIII. Antibacterial Proteins

Some of the ribosome inactivating proteins and antifungal proteins exhibit antibacterial activity. Two RIPs have been isolated and characterized from the roots of the Andean crop *Mirabilis expansa* radiobacter, *A. tumefaciens Pseudomonas* syringae (Vivanco et al., 1999).

Some pathogenic bacteria such as *Shigella dysenteriae* and certain strains of *E. coli.* produce type 2 RIPs called Shiga toxins that are made up of a single A chain and a pentamer of receptor-binding B chains Shiga toxins display an IC_{50} value of 0.8 nM against *E. coli* ribosomes (Suh et al., 1998).

Future perspectives and conclusions

Some of the antifungal proteins and peptides have been shown to possess translation-inhibiting, immunomodulatory (Wang and Ng, 2000a; Ye et al., 2001a,b, 2002c) and HIV-1 enzyme inhibiting (Ng et al., 2002) activities, just like ribosome inactivating proteins. Ribosome inactivating proteins also exhibit antifungal activity albeit weaker than that of other antifungal proteins.

In the foregoing account an impression can be gathered that antifungal proteins and peptides of different structures are produced by plants, animals, fungi and bacteria. Some of the antifungal proteins inhibit enzymes essential to the life cycle of human immunodeficiency virus (Ng et al., 2002). Table 1 presents the N-terminal sequences of some of the antifungal and antiviral proteins reported in the literature. Different antifungal proteins and peptides may exhibit different specificities in their antifungal action, and may differ in their inhibitory potencies toward the same fungus. Continued quest for more antifungal proteins and peptides

may lead to the discovery of molecules with potent activity against a broad spectrum of fungal species. Transgenic plants expressing the genes of these antifungal proteins and peptides may acquire a stronger resistance against fungal pathogens. The economic losses caused by fungal destruction of crops can thus be reduced.

Ribosome inactivating proteins represent an important group of antiviral proteins. The distinguishing feature of ribosome inactivating proteins is their RNA N-glycosidase activity that accounts for their translation-inhibiting activity. As noted above, RIPs and antifungal proteins overlap in their biological activities to some extent. Both type 1 and type 2 RIPs manifest antiviral activity. It awaits to be elucidated whether ribosome inactivating peptides (Parkash et al., 2002) and the recently reported new classes of RIPs (Lam et al., 1998; Ng and Parkash, 2002) also display antiviral activity. Nevertheless, type 1 RIPs are effective against a variety of animal and plant viruses. With the emergence of deadly viruses such as the avian flu virus and SARS virus, more research on antiviral therapeutic agents including antiviral proteins is needed to protect the lives of humans and their poultry.

Microorganisms including fungi, viruses and bacteria may threaten human health and the lives of livestock. Research on antimicrobial proteins will hopefully provide solutions to some of these problems.

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9 Compounds with Antioxidant Activity from Herbs

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Introduction

Oxygen-derived free radicals bring about lipid peroxidation which contributes to atherosclerosis, and are involved in myocardial damage seen in ischemia and reperfusion. However, some synthetic antioxidants may have toxic and/or mutagenic effects. Naturally occurring antioxidants with little or no toxicity are thus preferred. The aim of this chapter is to give an account of the variety of compounds with antioxidant activity as well as herbs with such activity, but whose active principles have not yet been chemically identified. Antioxidant activity in the studies referred to below was assayed in a number of ways including inhibition of lipid peroxidation (malondialdehyde formation) (Ahn et al., 2002; Morioka et al., 2004), inhibition of low density lipoprotein oxidation (Chu et al., 2003), and scavenging of superoxide and hydroxyl radicals (Osato et al., 1993; Shao et al., 2004).

Anthocyanins from dried flowers of *Hibiscus sabdariffa* L.

Pigments of *Hibiscus sabdariffa* reduced tert-butyl hydroperoxide-induced cytotoxicity in rat primary hepatocytes and diminished hepatotoxicity in rats. The activities of alanine and asparate aminotransferases in the serum declined and the incidence of liver lesions fell (Wang et al., 2000).

The ethyl acetate-soluble extract of *H. sabdariffa* dried flowers exhibited the highest free radical scavenging activity. The chloroform-soluble extract

manifested the most potent xanthine oxidase inhibitory activity. The residual fraction showed little activity. The first two fractions reduced the oxidative damage induced by tert-butyl hydroperoxide in rat primary hepatocytes as evidenced by malondialdehyde formation (Tseng et al., 1997).

Arbutin (4-hydroxyphenyl-β glucopyranoside, $C_{12}H_{16}O_7$) (Fig. 1)

Arbutin was the major antioxidant compound in the aqueous extract of *Rhodococcum vitis*-(L.) Avrorin. It inhibited the increase in serum alanine aminotransferase and glutathione S-transferase activities and lipid peroxidation in liver homogenate induced by galactosamine (Myagmar et al., 2004).

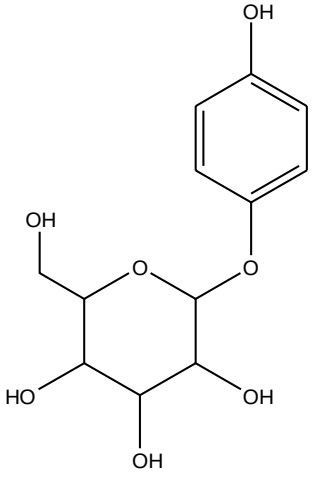

Fig. 1. Chemical structure of Arbutin.

Benzophenanthridine alkaloids

Vavreckova et al. (1996) reported the inhibition of 5- and 12-lipoxygenase by *Chelidonium majus* benzophenanthridine alkaloids.

Carnosic acid and carnosol (Fig. 2)

Carnosic acid from rosemary (*Rosmarinus officinalis* L.) scavenged free radicals as measured by the 2,2-diphenyl-1-picryhydrazyl assay and inhibited linoleic acid oxidation as measured by the beta-carotene assay (Wellwood and Cole, 2004). The use of ultrasound for extraction of carnosic acid using butanone, ethyl acetate and ethanol as solvents improved the

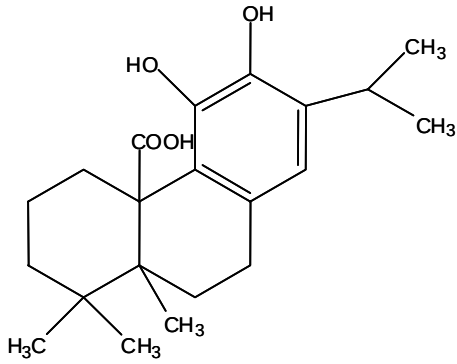

Fig. 2. Chemical structure of Carnosic acid.

yield, reduced the extraction time, and increased the extraction efficiency of poor solvents like ethanol. The ethanolic extract of dried herb was more efficient than that of fresh herb owing to the presence of water in the latter (Albu et al., 2004).

Carnosol induced, at the level of mRNA as well as protein, the expression of heme oxygenase-1 that protects cells against toxins. The antioxidant activity of carnosol was partly inhibited by phosphatidylinositol 3-kinase (PI3K) or heme oxygenase-1 inhibitors, suggesting that it acts through a pathway involving (PI3K) or heme oxygenase 1 (Martin et al., 2004).

Coumarins (2H-1-Benzopyran-2-one, $C_9H_6O_2$)

Scoparone (6,7-dimethoxycoumarin) (Fig. 3) from the hypolipidemic Chinese herb *Artemisia scoparia* scavenged reactive oxygen species. This activity at least partially explains its retarding effect on pathological changes in hypercholesterolemic rabbits with diabetes (Hoult and Paya, 1996).

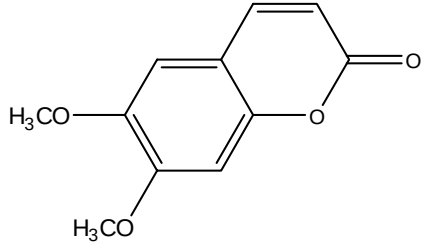

Fig. 3. Chemical structure of Scoparone.

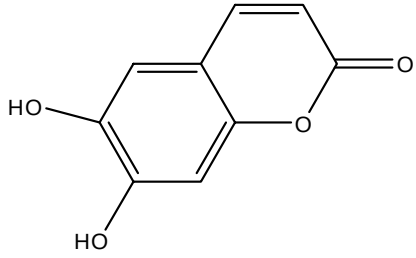

Fig. 4. Chemical structure of Esculetin.

Esculetin (6, 7-dihydroxycoumarin, $C_aH_6O_4$) (Fig. 4) from *A. scoparia* is a lipoxygenase inhibitor. It inhibited Ras-mediated cell proliferation and attenuates vascular restenosis after angioplasty in rats (Pan et al., 2003). Esculetin from *Alchemilla speciosa* had antimutagenic activity (Schimmer and Eschelbach, 1997).

The coumarin xanthotoxol from *Cnidium monnieri* fruits had potent antioxidant activity. On the other hand, columbianetin, bergapten and angelicin from *Cnidium monnieri* fruits had only slight antioxidant activity (Ng et al., 2000).

Ecdysteroids

Ecdysone (Fig. 5) is (2β, 3β, 5β, 20R)–2, 3, 14, 20, 22, 25 hexahydroxycholesten-7-en-6-one. Ecdysteroids possess an ecdysone skeleton.

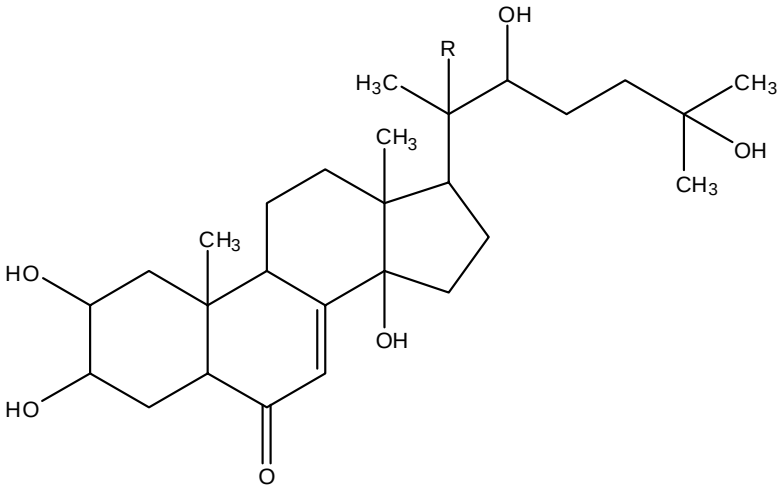

Fig. 5. Chemical structure of α-ecdysone.

From the Chinese herb *Serratula strangulata*, four ecdysteroids including 20-hydroxyecdysone (E1), 25-deoxy-11,20-dihydroxyecdysone (E2), 24-(2-hydroxyethyl)-20-hydroxyecdysone (E3) and 20-hydroxyecdysone-20,22-monoacetonide (E4) were isolated. They protected human erythrocytes against oxidative hemolysis induced by a water-soluble inhibitor, 2,2'-azobis 2-aminopropane hydrochloride, and inhibited rat hepatic microsome peroxidation (Cai et al., 2002).

Flavonoids

Flavonoids are polyphenolic compounds found in many fruits and vegetables. Flavonoids (apin, luteolin-, apigenin-glycosides) and essential oils (apiol, miriszticin) are components of the parsley (*Petroselinum crispum* (Mill.) Hill) fruit that contribute to its antioxidant activity (Fejes et al., 1998).

Baicalein (5,6,7-trihydroxyflavone) from the root of the Chinese herb *Scutellaria baicalensis* Georgi exhibited antioxidant, anti-inflammatory and 12-lipoxygenase inhibitory activities. It selectively inhibited the nitric oxide-dependent apoptotic pathway of activated brain microglia by suppressing production of cytotoxic nitric oxide (Suk et al., 2003). Baicalin inhibited production of eotaxin, an eosinophilic chemokine associated with eosinophil recruitment to allergic inflammation sites (Nakajima et al., 2001).

Purearin (8-(β-D-glucopyranosyl-7-hydroxyl-3-(4-hydroxyphenyl-4H-1-benzopyran-4-one) (Fig. 6) is the main flavonoid in the root of the wild leguminous creeper *Purearia lobatai*. Both purearin and the crude extract of *P. lobata* roots inhibited the steady-state chemiluminscent reaction based on horseradish peroxidase and a luminol-oxidant-enhancer reagent, albeit with different mechanisms, suggesting the presence of active compounds other than puararin in the crude extract. The crude extract showed strong antioxidant activity (Guerra et al., 2000).

Firenzuoli et al. (2004) reported that dietary consumption of flavonoids

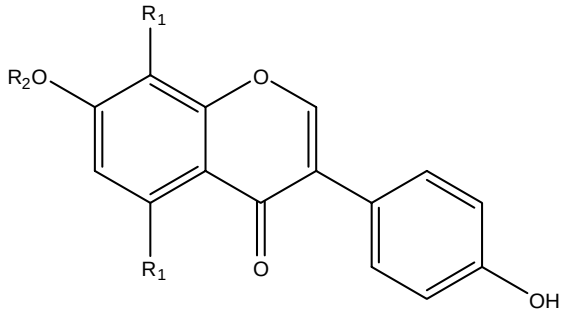

Fig. 6. Chemical structure of Purearin.

had an inverse relationship with the later development of cancer and coronary heart disease and that citrus species, *Ginkgo biloba*, hawthorn, soy, St. John's Wort, *Silybum marirum*, tea and *Vaccinium mirtillis* contained flavonoids. Kolaviron, a biflavonoid fraction of *Garcinia kola*, reduced oxidative damage to DNA in the liver (Farombi et al., 2004). The antioxidant principles in the Nigerian herb *G. kola* that inhibited lipid peroxidation in rat liver homogenate were tentatively identified as isoflavones (Adegoke et al., 1998).

The flavonoids baicalin, luteolin-7-glucuronide-6'-methyl ester and rutin from *S. baicalensis, Ixeris denticulate f. pinnatipartia* and *Sophora japonica,* respectively, had potent antioxidant activity. In contrast, naringin from *Drynaria fortunei* rhizomes had only low activity (Ng et al., 2000).

Gallic acid (Fig. 7)

The structure of catechin is (2R, 3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3.5.7-triol (Fig. 8).

Epigallocatechin-3-gallate (EGCG), which is (2R3R)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol 3-(3,4,5-

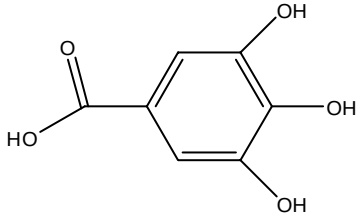

Fig. 7. Chemical structure of Gallic acid.

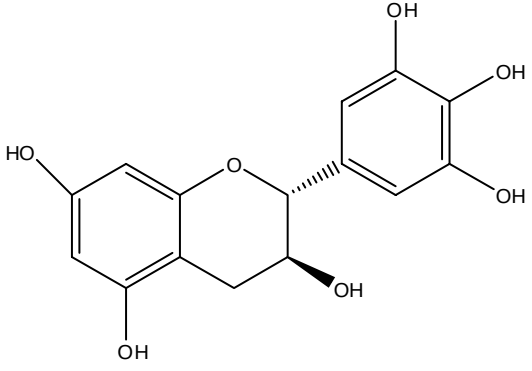

Fig. 8. Chemical structure of Catechin.

trihydroxybenzoate), a constituent of green tea, inhibited proliferation of cultured rabbit lens epithelial cells by inducing apoptosis. The findings opened up the possibility of using EGCG for the prevention of postcapsular opacity (Huang et al., 2000).

Gallic acid found in the medicinal herb *Limonium wrightii* from the Okinawa islands scavenged 1,1-diphenyl-2-picrylhydrazyl, superoxide anions and hydroxyl radicals. It suppressed pH or bol-12-myristate acetate-stimulated production of reactive oxygen species in polymorphonuclear leukocytes. It also inhibited lipid peroxidation in rat liver microsomes and reduced CCl_4-induced hepatotoxicity (Aniya et al., 2002).

One of the antioxidant compounds isolated from the aqueous extract of dried rose (*Rosa rugosa* Thunb.) flowers by ion exchange chromatography on CM-cellulose and gel filtration on Sephadex G75 was a derivative of gallic acid (3,4,5-trihydroxybenzoic acid). Another two included a polysaccharide and a polysaccharide-peptide gallic acid, which demonstrated the most potent antioxidative activity.

Gypenosides

Gypenosides, (Fig. 9) which are saponins (steryl glycosides) from the Chinese herb *Gynostemma pentaphyllum*, reduced the content of superoxide anions and H_2O_2 in human neutrophils, and inhibited chemical-induced lipid peroxidation in liver microsomes and vascular endothelial cells. The pigments also reduced the oxidative stress in human monocytes and murine macrophages (Li et al., 1993).

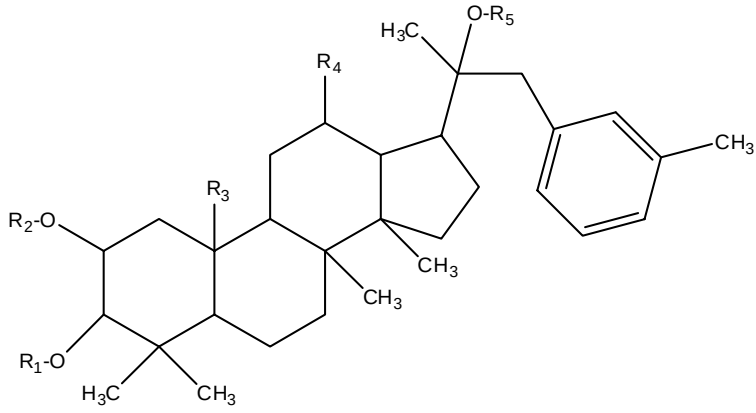

Fig. 9. Chemical structure of dammarane-type gypenosides (where R1 = glucose, rhamnose; R2 = glucose, rhamnose, etc.; R3 = CH_3, CH_2OH, CHO; R4 = H, CH_2OH; and R5 = glucose, xylose, etc.

Hamamellitannin (Fig. 10)

It is the major active component of *Hamamelis virginiana* L. (witch hazel) bark. It demonstrated potent peroxynitrite scavenging activity. Peroxynitrite is cytotoxic with oxidizing activity toward nucleotides, amino acids and lipids, and causes lipid peroxidation, cell death, carcinogenesis and aging (Choi et al., 2002).

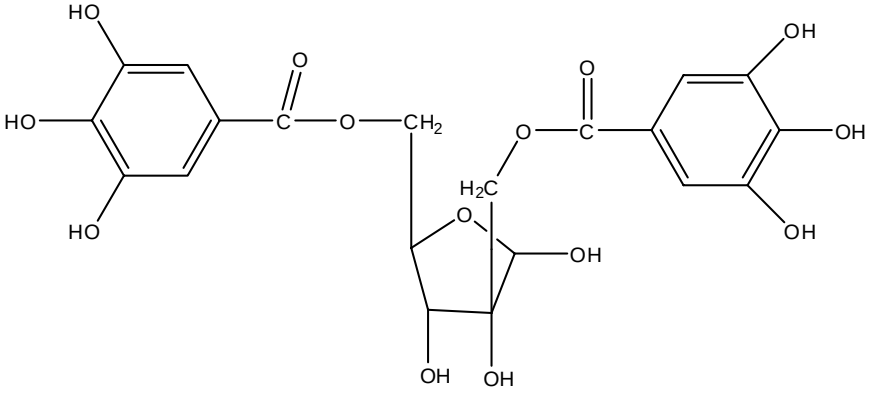

Fig. 10. Chemical structure of Hammamelitannin (2′,5-di-O-galloyl hamamelose).

Honokiol

Honokiol from the Chinese herb *Magnolia officinalis* potently inhibited lipid peroxidation in rat heart mitochondria and liver mitochondria (Chiu et al., 1997).

4-Hydroxy-5-hydroxymethyl-[1,3]dioxolan-2, 6′-spiran-5′, 6′, 7′, 8′-tetrahydro-indolizine-3′-carbaldehyde (HDTIC)

Two isomers of HDTIC were isolated from *Astragalus membranaceus* (Fisch). Both delayed replicative senescence of human fetal lung diploid fibroblasts. This activity was attributed to several factors, one of which was their antioxidant activity (Wang et al., 2003).

Magnolol

Magnolol ([1,1′-biphenyl]-2,4′-diol, 3′-5-di-2-propenyl) from the Chinese herb *Magnolia officinalis* inhibited the production of superoxide anions and the activity of mycloperoxidase, indices of neutrophil infiltration in the ischemic myocardium, suggesting that it might reduce myocardial

ischonia/reperfusion injury (Lee et al., 2001). Magnolol also attenuated monocyte chemotactic protein 1 expression and intima hyperplasia in the balloon–damaged aorta of cholesterol-fed rabbits, and vascular cell adhesion molecule-1 expression in tumor necrosis factor α-treated human aortic endothelial cells (Chen et al., 2002).

Kong et al. (2000) showed that magnolol, given either prior to or subsequent to rats after induction of sepsis by cecal ligation and puncture, reduced lipid peroxidation in lungs, liver and plasma of the septic rats, probably due to its antioxidant activity. The data suggest that magnolol may be useful for the treatment of sepsis (Kong et al., 2000).

Magnolol inhibited the proinflammatory cytokine response following hemorrhagic shock and resuscitation in rats. This might lead to reduced tissue damage and increased survival during subsequent intra-abdominal sepsis (Shih et al., 2003).

Phenylethanoid glycosides (PEG)

Four PEG (Fig. 11) have been isolated from the Chinese herb *Brandisia hancei*. These comprised acteoside (=β-(3,4-dihydroxyphenyl)-ethyl]-(3′-0-α-L-rhamnopyronosyl)-(4′-0-caffeoyl)-β-D-glucopyranoside), acetylacteoside, poliumoside (=β-(3,4-dhydroxyphenyl)-ethyl)-(3′, 6′-0-α-L-dirhamnopyranosyl)-(4′0-caffeoyl)-β-D-glucopyranoside) and brackeoside. The last two demonstrated stronger free radical scavenging effects and more potently inhibited free-radical induced hemolysis (He et al., 2000).

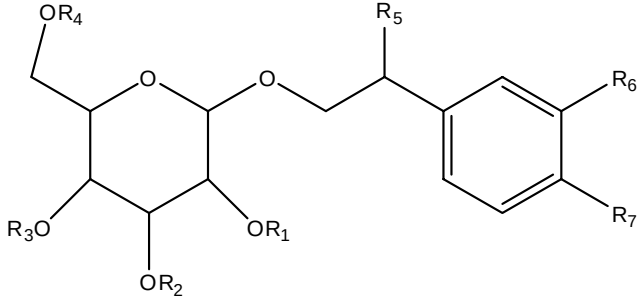

Fig. 11. Chemical structure of Phenylethanoid glycoside.

Phenylpropanoid glycosides (PPG)

PPG isolated from the Chinese herb *Pedicularis striata* inhibited Fe^{2+} dependent lipid peroxidation through chelation of Fe^{2+} ions and under physiological conditions the chelates formed are sufficiently stable (Li et al., 1997).

Proanthocyanidins from grape seeds

Grape seed proanthocyanidins and *Scutellaria baicalensis* were synergistic in their ability to scavenge reactive oxygen species (Shao et al., 2004). Thus lower dosages of each of them can be used and possible side effects due to high dosages can be avoided.

Salvianolic acid B

Salvianolic acid B from the Chinese herb *Salvia miltiorrhiza* and the aqueous methanolic extract of *S. miltiorrhiza* attenuated the expression of vascular adhesion molecule-1 and intercellular cell adhesion molecule-1 in human aortic endothelial cells treated with tumor necrosis factor-alpha. This activity, together with their anti-inflammatory and low density lipoprotein-inhibiting activities, may explain their usefulness for the treatment of atherosclerosis. All aforementioned activities are related to their antioxidant activities (Chen et al., 2001). A salvianolic acid B-enriched fraction of *S. miltiorrhiza* induced neointimal cell apoptosis in a rabbit angioplasty model (Hung et al., 2001).

Schisandrin B (Fig. 12)

It is a dibenzocyclooctadiene derivative from *Fructus schisandrae*. It had protective effects against cerebral oxidative stress (Ko and Lam, 2002).

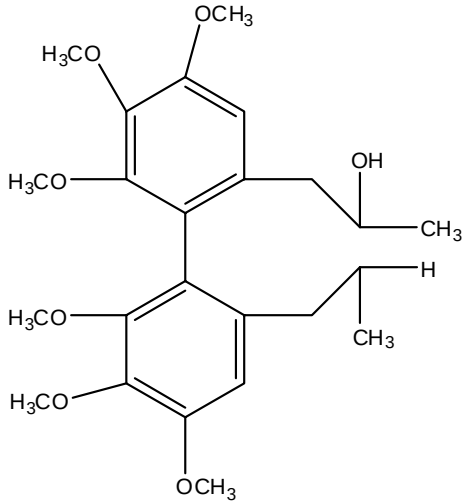

Fig. 12. Chemical structure of Schisandrin B.

Sodium tanshinone sufonate

It is a derivative of tanshinone IIA (=1,6-dimethylphenanthro(1,2-b) furan-10, 11-dione) isolated from *S. miltiorrhiza* rhizomes. It reduced the size of myocardial infarcts, and prolonged the survival of cultured human saphenous vein endothelial cells but not human ventricular myocytes *in vitro* upon exposure to xanthine oxidase-generated oxyradicals. The data suggest that it may protect the vascular endothelium (Wu et al., 1993).

Tetrandrine (Fig. 13)

It is an alkaloid (+)-(S)-3-phenyl-1′-(phenylmethyl)-(3,4′-bipperidine)-2,6-dione-HCl, isolated from the Chinese herb *Stephania tetrandra*, its free radical scavenging activity contributed to its inhibitory activity on nuclear factor-kappa B activation by phorbol 12-myristate 13-acetate (Ye et al., 2000).

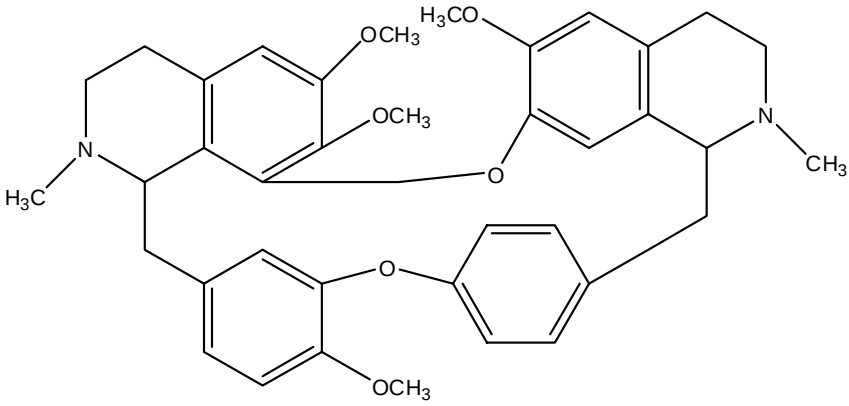

Fig. 13. Chemical structure of Tetrandrine.

Thonningianin

Two ellagitannins, designated as thonningianins A and B, were isolated from the African herb *Thonningia sanguinea*. They exhibited potent free radical scavenging activity against 1,1-diphenyl-2picryhydrazyl as evidenced by electron spin resonance analysis (Ohtani et al., 2000). Thonningianin A inhibited human glutathione S-transferase, an enzyme involved in resistance of tumor cells against chemotherapeutics (Gyamfi et al., 2004).

Trilinolein ($C_{57}H_{98}O_6$)

Trilinolein (Fig. 14), which is (1β)-6,6′,7,12-tetramethoxy-2,2′-dimethyl-berbaman isolated from sanchi ginseng (*Panax notoginseng*), had myocardial protective effects related to its antioxidant activity (Chan and Tomlinson, 2000). It reduced norepinephrine-induced protein synthesis via inhibition of cardiomyocyte production of reactive oxygen species (Liu et al., 2004a). Angiotensin II-stimulated protein synthesis, beta-myosin heavy chain promoter activity, intracellular reactive oxygen species production, and H_2O_2-activated phosphorylation of mitogen-activated protein kinases, were all inhibited by trilinolein and the antioxidant N-acetylcysteine (Liu et al., 2004b). Trilinolein counteracted the free radical damage associated with atherogenesis, and ischemial reperfusion induced myocardial damage. The findings constitute a scientific basis for the traditional use of *Panax notoginseng* for the treatment of circulatory problems (Chan et al., 2002). Trilinolein increased the activity of superoxide dismutase in cultured rat brain astrocytes (Chiu et al., 1999). It preserved heart mitochondrial ultrastructure after exposure to ischemia and reduced oxygen free radical production by leukocytes (Chan et al., 1996).

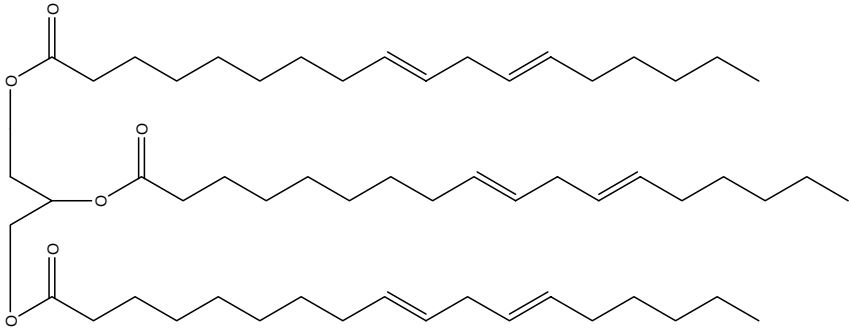

Fig. 14. Chemical structure of Trilinolein.

Other compounds

The lignan 4′-demethyldeoxypodophyllotoxin, the alkaloid tetrahydropalmatine (5,8,13,13a-tetrahydro-2,3,9,10-tetramethoxy-6H-dibenzo [a, g] qurnolizine) and the bisbenzyl erianin (2-methoxy-5-[2-(3,4,5-trimethoxy-phenyl)-ethyl]-phenol) from *Sinopodophyllum emodii* (Wall) Ying, *Stephania sinica* Diels and *Dendrobium chrysotoxum* Lindl respectively exhibited potent antioxidant activity in both lipid peroxidation and hemolysis assays. The terpene tanshinone I from *S. miltiorrhiza* inhibited lipid peroxidation but not hemolysis (Ng et al., 2000).

Different compounds from *Aster tataricus* Linn. f.

A number of compounds were isolated from the medicinal plant *Aster tataricus* including shionone, epifriedelinol, quercetin 2-(3,4-dihydroxyphenyl-3,5,7-trihydroxy-chromen-4-one), (Fig. 15) kaempferol (3,5,7-trihydroxy-4′-methoxyflavone) (Fig. 16), scopoletin (7-hydroxy-6-methoxy-2H-chromen-2-one) (Fig. 17), emodin (3-methyl-1,6,8-trihydroxy-anthraquinone) (Fig. 18), aurantiamide acetate (N-benzoyl-phenylalanyl-phenylalaninol acetate) and 1,7-dihydroxy-6-methyl-anthraquinone. The compounds were compared with regard to their ability in inhibiting hemolysis of rat erythrocytes induced by 2′-2′ azobis (2-amidinoproane) dihydrochloride, lipid peroxidation using a $FeSO_4$-ascorbic acid system, and generation of superoxide radicals using a phenazine methosulfate-

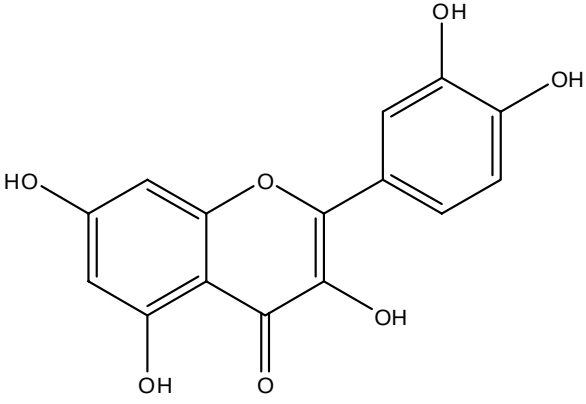

Fig. 15. Chemical structure of Quercetin.

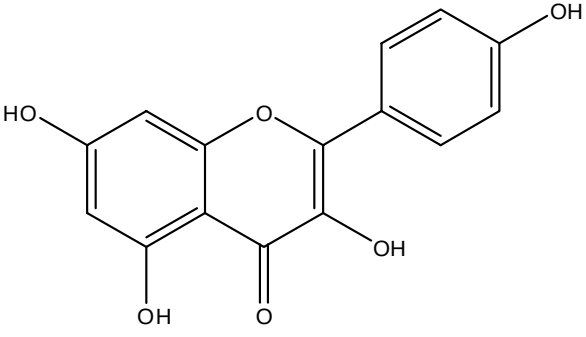

Fig. 16. Chemical structure of Kaempferol.

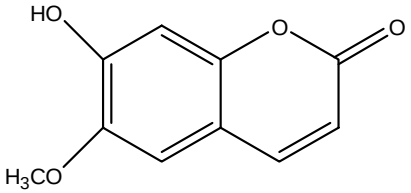

Fig. 17. Chemical structure of Scopoletin.

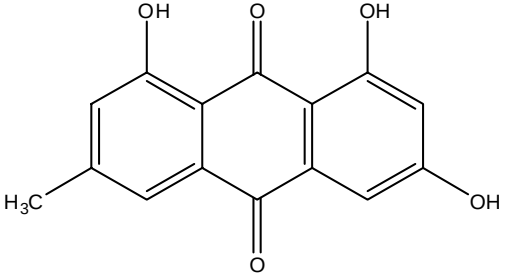

Fig. 18. Chemical structure of Emodin.

nicotinamide adenine dinucleotide system. Effects on the Fe-bleomycin-induced DNA damage reflected pro-oxidant activity. Quercetin and kaempferol were most potent in inhibiting hemolysis, lipid peroxidation, and superoxide radical generation. Scopoletin and emodin were similar to quercetin and kaempferol in inhibiting superoxide radical generation and second to them in inhibiting lipid peroxidation. Aurantiamide acetate exhibited some inhibitory activity toward superoxide radical generation. 1,7-dihydroxy-6-methyl-anthraquinone exerted an inhibitory activity only on superoxide radical generation. Shionone and epifriedelinol did not display any antioxidant activity. Quercetin and kaempferol, but not the remaining compounds, exhibited some pro-oxidant activity (Ng et al., 2003).

Herbal extracts

Achyrocline satureoides (Lam.) DC.

The aqueous extract of this South American herb inhibited the oxidation of human low density lipoprotein (Gugliucci and Menini, 2002). Both aqueous and methanolic extracts inhibited the generation of thiobarbituric acid-reactive substances in rat liver homogenate (Desmarchelier et al., 1998).

Allium sativum L.

Garlic had potent antioxidant activity. In addition, it could prevent cardiovascular disease, cancer, arthritis, cataract formation and thrombus formation (Rahman, 2003).

Anthrisus cerefolium (chervil)

An aqueous extract demonstrated anti-liperoxidant and antioxidant activities (Fejes et al., 2000).

Arctium lappa L.

The herb had hepatoprotective activity perhaps due to its antioxidative effect (Lin et al., 2000).

Artemisia campestris L.

An ethanolic extract of the leaves of this Indian plant had a wound-healing effect in rats and at the same time produced an increase in the activity of the antioxidant enzymes, catalase and superoxide dismutase, in granuloma tissue (Shirwaikar et al., 2003). A water extract of the herb potently scavenged 1,1-diphenyl-2-picrylhydrazyl, hydroxide and superoxide anion radicals (Aniya et al., 2000).

Astragalus erianthus

It exerted antioxidant effects in patients receiving therapy against pulmonary tuberculosis (Skakun and Blikhar, 1986).

Astragalus membranaceus var. *mongholicus*

The antioxidant activity of this Chinese herb accounted for its cardioprotective effect and its therapeutic benefit in treatment of myocardial infarction, heart failure and angina pectoris (Miller, 1998).

Carica papaya L.

The pulp and seeds of unripe papaya had the ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl, hydroxyl and superoxide radicals. Citric acid, glucose, malic acid and vitamin C possibly contributed to the antioxidant activity (Osato et al., 1993).

Crataegus pinnatifida var. psilosa fruits

This is a medicinal herb used for making a soft drink in Taiwan. The hot water extract of its dried fruits are composed of flavonoids (6.9%), procyanidins (2.2%), catechin (0.5%) and epicatechin (0.2%), chemical structures are very common and can be found in many papers, exhibited an antioxidant action (Chu et al., 2003).

Curcuma longa L.

Its antioxidant activity, together with its other activities including anti-inflammatory, anticarcinogenic and antimicrobial activities, have been reviewed (Anonymous, 2001a).

Desmotrichum fimbriatum Blume

The aqueous ethanolic extract of this ayurvedic herb ameliorated the peroxidative damage caused in mice by cold water swim stress (Chakrabarty et al., 2001).

Dimocaepus longan L.

An aqueous extract of the herb demonstrated hydroxyl radical-scavenging activity as revealed by a gas chromatography-mass spectrometry method based on the Fenton reaction system (Wang and Smythe, 2003).

Drynaria fortumei [Kunze] J. SM

The water extract of this Chinese herb lowered the level of intracellular reactive oxygen species in rat osteoblasts, and protected osteoblasts from H_2O_2-induced death (Liu et al., 2001).

Echinacea spp.

It had antioxidant as well as anti-inflammatory activities (Rininger et al., 2000).

Epimedium spp.

Its extract increased erythrocyte and hepatic soperoxide dismutase activity and erythrocyte glutathione peroxidase activity, while it reduced hepatic lipoperoxide and cardiac muscle lipofuscin in aged animals (Zeng et al., 1997).

Fructus corni (*Cornus officinalis* SIEB et Zucc.)

It reduced oxidative stress in macrophages and endothelial cells (Peng et al., 1998).

Ginkgo biloba L.

Its extract inhibited tumor necrosis factor α-induced reactive oxygen species production, transcription factor activation, and cell adhesion molecule in human aortic endothelial cells (Chen et al., 2003). *Ginkgo biloba* is known for improving mental sharpness by increasing blood flow to the brain (Gajewski and Hensch, 1999).

Juglans sinensis (D.C.) DODE

Ahn et al. (2002) found that *J. sinensis* extract exerted a protective effect against $HgCl_2$-induced acute renal failure. This effect might be due to its antioxidant activity since it counteracted lipid peroxidation in renal cortical slices brought about by $HgCl_2$.

Mesona procumbens Hemsl.

The antimutagenic activity of an aqueous extract of *M. procumbens* might be caused by polyphenolic compounds and ascorbic acid (Yen et al., 2001).

Panax ginseng C. A. Meyer

Its major active constituents, the ginsenosides, (steryl glycosides), displayed antioxidant activity (Keifer and Pantuso, 2003).

Peucedanum japonicum Thunb.

This Japanese herb exhibited antioxidant and antiproliferative activities. Its preventive effect on the initial stage of colon carcinogenesis is related to inhibition of the incidence of aberrant crypt foci and β-caterin accumulated crypts by scavenging of free radicals in the colon mucosa, and to the modulation of cell proliferation in premalignant lesions. (Morioka et al., 2004).

Picrorhiza kurroa Royle ex Benth

This Ayurvedic herb possessed antioxidative activity among other activities such as hepatoprotective, immunomodulating and anticholestalic activities

(Anonymous, 2001b). Apocynin 1-(4-hydroxy-3-methoxyacetophenone) from its roots inhibited platelet aggregation and production of inflammatory mediators by lung macrophages (Engels et al., 1992).

Pluchea sagittalis (Lam.) Cabera

The aqueous extract of this South American plant exerts a potent antioxidant action on blood leukocytes and exhibits strong anti-inflammatory activity (Perez-Garcia et al., 1996).

Podophyllum hexandrum (Syn. *P. emodi*.)

It is a Himalayan herb with antitumor and radioprotective activities. Preirradiation treatment with the herb produced a rise in intestinal superoxide dismutase activity but no changes in hepatic catalase activity in the postirradiation period. The herb had a protective action upon exposure to lethal whole-body irradiation (Mittal et al., 2001).

Polygonum multiflorum var. *hypoleucum*

The antimutagenic activity of its root extract has been demonstrated by the Tradescantia micronucleus assay. This activity was ascribed to the antioxidant action of *P. multiflorum* enhancement of DNA repair or radical elimination from irradiated cells (Zhang et al., 1999).

Rhaponticum uniflorum (L.) DC.

This Chinese herb inhibited lipid peroxide generation and improved membrane fluidity of smooth muscle cells. It also made atherosclerotic lesions less severe (Lu, 1993).

Rhodiola rosea L.

This plant had adaptogenic characteristics. Its biologically active constituents, also mainly found in the rhizome, included rosarin, rosarin, rosin, salidroside and tyrosol. All showed some antioxidant activity and prevented the cardiovascular system from stress and arrhythmias (Kucinskaite et al., 2004). *Rhodiola* reduced hypoxia-induced oxidative injury (Ip et al., 2001).

Rhubarb (*Rheum officinale* Baillon)

Iizuka et al. (2004) have shown the antioxidant activity of rhubarb constituents toward low density lipoprotein.

Rubus chingi (j)

An aqueous extract of its fruits protected primary rat liver cells against tert-butyl hydroperoxide induced oxidative stress (Yau et al., 2002).

Salvia lavandulaefolia Vahl. (Spanish sage)

Antioxidant activity, as evidenced by inhibition of bovine brain liposome peroxidation, was detected in the extract (Perry et al., 2001).

Salvia miltiorrhiza var. *miltiorrhiza*

Xia et al. (2003) observed that antioxidant therapy with a crude extract of *S. miltiorrhiza* reduced the rise of plasma endothelin-1, thromboxane B2 and malondialdehyde after cardiopulmonary bypass in patients with congenital heart disease. An extract of *S. miltiorrhiza* increased vitamin E content of LDL and reduced atherosclerosis in cholesterol-fed rabbits (Wu et al., 1998).

Salvia officinalis L. (sage)

Its antioxidant activity was reported by Dauksas et al. (2001).

Serratula spp.

Bathori et al. (2004) demonstrated the inhibitory effect of aqueous methanol extracts of *S. coronata, S. tinctoria* and *S. wolffii* on lipid peroxidation. The flavonoid-containing fraction of S. *coronata* had a higher inhibitory activity than the corresponding ecdysteroid-containing fraction.

Sutherlandia frutescens L.

This South African herb is used to treat cancer by the natives and recently to treat AIDS patients aiming at improving their health, demonstrated antioxidant activity (Tai et al., 2004). This African medicinal herb *Thonningia sanguinea* contains thonningianin A which has antioxidant activity (Gyamfi and Aniya, 2002).

Withania somnifera L.

This Ayurvedic herb exhibited antioxidative as well as a host of other activities including anti-inflammatory, antitumor, anti-stress, immuno-modulating, hemopoietic and rejuvenating activities (Mishra et al., 2000).

Other herbs

Zhu et al. (2004) reported that many traditional herbs used for treating cardiovascular disorders, including *Camelia sinensis, Herba leonuri,* Radix *Achyranthis bidentate, Rhizoma ligustici* and *Salvia miltiorrhiza* contained nonphenolic compounds that could inhibit oxidative reactions mediated by the inflammatory oxidants, peroxynitrite, hypochlorous acid and hydroxy radical and also iron-dependent lipid peroxidation.

Bartlett and Eperjesi (2004) revised the use of the following nutrients in ocular disease: *Ginkgo biloba*, the carotenoids, vitamins A, B, C and E, minerals zinc and selenium, lutein and zeaxanthin. The nutrients are known to have antioxidant activity or are associated with antioxidant enzymes. The inclusion of vitamins C and E and lutein/zeaxanthin in nutritional supplement was recommended.

Farombi et al. (2004) noted that black currant juice displayed antioxidant activity that could not be entirely accounted for by the presence of vitamin C.

Ichikawa et al. (2003) showed that shengmai san, a traditional Chinese medicine formula composed of *Panax ginseng, Ophiopogon japonicus* and *Schisandra chinensis*, was able to prevent cerebral oxidative damage in rats.

The antioxidative activity of aqueous infusions of sage (*Salvia officinalis* L.) originated from both polyphenolic and nonpolyphenolic substances (Matsingou et al., 2003).

Chinese red yeast rice displayed anti-atherosclerotic activity (Heber et al., 2001).

Akamatsu et al. (1997) studied the antioxidant action of keigai-rengyo-to, an oral Japanese Kampo (herb) medicine on neutrophil functions. The results indicated that its clinical efficacy on acne may be partly attributed to its antioxidant action on infiltrated neutrophils.

Kahkonen et al. (1999) reported high antioxidant activity that correlated with a high phenolic content in berries, especially crowberry and aronia. Apple extracts exhibited potent antioxidant activity despite a low phenolic content. The medicinal plants bog-rosemary, heather, meadowsweet, and willow herb, and the nonedible plant materials including birch phloem, pine bark and cork, spruce needles and willow bark showed high antioxidant activity. Beetroot peel and potato peel also demonstrated potent antioxidant activity.

Liu and Ng (2000) examined aqueous extracts of 12 Chinese herbs for antioxidant activity, including *Andrographis paniculata, Arctium lappa, Coptis chinensis, Fritillaria cirrhosa, Lonicera japonica, Paeonia suffruticosa, Paris chinensis, Pinella ternata, Prunella vulgaris, Prunus persica, Senecio scandens*

and *Sophora flavescens*. The extracts of *C. chinensis, P. suffruticosa, P. vulgaris* and *S. scandens* were the most potent in inhibiting rat erythrocyte hemolysis and in scavenging superoxide and hydroxyl radicals.

Conclusions and Future Perspectives

Many herbs exhibit antioxidant activity. In some cases the chemical structure of the active principle has been elucidated. In addition to antioxidant activity, the compounds may possess related activities such as anti-inflammatory and cardioprotective activities. The antioxidant constituents in many herbs remain to be chemically characterized and it is likely that some of these compounds are novel. The bulk of results from animal experiments indicate the beneficial effects of antioxidants on the health status although data regarding human populations, including those arising from multi-national collaborative efforts that involved studies of different centers, are less unequivocal. Nevertheless, intake of antioxidants such as vitamins C and E and melatonin as dietary supplements and foods and herbs rich in antioxidants such as kiwi fruits, blueberries, strawberries and nuts may protect against some diseases such as cardiovascular, neurodegenerative and malignant diseases.

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10 Bioactivity of Medicinal Plants: Progress and Perspectives

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Abbreviations

AIDS (acquired immune deficiency syndrome); COX (cyclooxygenase); ER (estrogenic receptor); HBsAg (hepatitis B surface antigen); HBV (hepatitis B virus); HCV (hepatitis C virus); HIV (human immunodeficiency virus); ICAM-1 (intercellular adhesion molecule-1); IFN-γ (interferon-γ); LDL (low-density lipoprotein); LOX (lipoxygenase); LTB_4 (leukotriene B_4); NF-κB (nuclear factor-κB); NO (nitric oxide); NOS (nitric oxide synthase); PGE_2 (prostaglandin E_2); PAP (pokeweed antiviral protein); PG (prostaglandin); TGF-β (transforming growth factor-β); TNF-α (tumor necrosis factor-α); TXB_2 (tromboxane B_2).

Introduction

Are medicinal plants effective in the treatment of human diseases? The answer is a resounding 'yes' The ancestral use of medicinal plants to treat different pathologies is, in itself, a strong argument for phytotherapy. As a scientific field, however, the study of medicinal plants has often given rise to misinformation as some of the observed results of treatment with these substances may, in fact, be due to a placebo rather than a pharmacological effect. For this reason, the clear differentiation between the 'activities' and the 'effects' of medicinal plants against diseases and pathologies is essential for establishing the differences between the uses of medicinal plants in folk medicine, experimental pharmacological studies and clinical trials.

Folk medicine and medicinal plants

The use of medicinal plants for healing is as old as humankind itself. There are many historical references to the use of plants to treat or prevent afflictions and diseases, with countless documents confirming the use of medicinal plants in different countries, cultures and civilizations in Europe, Asia, America and Africa over the last 5000 years. But even prehistoric man knew about the healing powers of plants, as evidence from the archaeological excavations in Shanidian (Iraq) show. The findings there indicate that some 60,000 years before our civilization came to be, Neanderthal man already had a rudimentary knowledge of the use of medicinal plants. One of the most relevant documents on medicinal plants, however, dates from about 1555 BC and makes reference to about 160 crude drugs, their applications and the various prescriptions used during the first Egyptian dynasties from 3300 to 2600 BC (De Pasquale, 1984).

In Europe, knowledge concerning materia medica and medicinal plants evolved greatly in both the Greek and Roman eras, with Hippocrates, Galen and Dioscorides being the most representative figures, but the use of medicinal plants subsequently decreased, partly due to the new-gained knowledge of active principles and the birth of pharmacochemistry. The isolation of natural products from plants and the studies of Magendie and Claude Bernard on the mechanism of action of various drugs further reduced the interest in the therapeutic use of medicinal plants and their active extracts, relegating their use to popular remedies and folk medicine in some rural regions of the more developed countries. In Asia, however, the use of medicinal plants against diseases was maintained and improved upon with new developments in phytotherapy. It is perhaps for this reason that the two leading countries in phytotherapy today are China, with its world-renowned traditional medicine, and India, famous for its Ayurvedic medicine.

Natural products were, of course, the main focus of both physiology and pharmacology when these two sciences were in their initial phases. Atropine, cocaine, morphine, tubocurarine and other alkaloids allowed researchers to establish the physiological mechanisms of endogenous mediators and the mode of action of drugs. More recently, capsaicin and cannabinoids have been the object of mechanist studies which have revealed several previously unknown biochemical, physiological and pharmacological effects of these substances. Capsaicin, the pungent ingredient of chilli peppers, has thus proven to be an extremely relevant natural product as the study of this compound has shed light on the sensory pharmacology, physiology and biochemistry of the vanilloid receptor TRPV1 (Szolcsányi, 2004; Cortright and Szallasi, 2004). In the case of cannabinoids, the receptors CB1 and CB2 have become attractive targets

(Pavlopoulos et al., 2006) for designing therapeutic ligands for potential treatments for obesity and the prevention of comorbid metabolic disorders (Antel et al., 2006), gastrointestinal diseases (Massa and Monory, 2006), liver diseases (Mallat and Lotersztajn, 2006) and diabetes (Lebovitz, 2006).

Research in medicinal plant activity: past, present and future

Upon the discovery of natural products as active compounds from crude drugs and of their potential use in medicine, research on medicinal plants began to focus on medicinal chemistry in order to discover the potential active compounds of such products, along with their possible derivatives and the various structure-activity relationships. This type of research led to the synthesis of derivatives such as aspirin, which was widely used as an analgesic, an anti-inflammatory and, more recently, an anti-thrombosis agent. Over time, more and more synthetic drugs were introduced as a result of studies that focused on increasing the activity of known compounds while simultaneously reducing the non-desirable side effects and toxicity. In this way, drugs such as verapamil gave rise to a new group of active chemicals that served as relaxants of smooth muscle, which in turn led to another new generation of drugs, the calcium antagonists. Likewise, research on opioids produced new agonists and antagonists that were used as central analgesics; it also led to the isolation of monacholin K, later dubbed lovastatin, from the fungus *Monascus purpureus* Went, which then opened the way to a new class of hypocholesterolemiant drugs, the inhibitors of β-hydroxy-β-methylglutaryl CoA (HMG-CoA) reductase (Robbers and Tyler, 1999).

Clearly then, a great motivation for the increase in research on medicinal plants in the past few decades has been to obtain new compounds for use as drugs. In addition, other interesting studies have been carried out to gain insight into the pharmacological activity of plant extracts. Thus, numerous research groups all over the world have studied the antimicrobial, anticancer, anti-inflammatory, immunostimulant and other activities of extracts from medicinal plants. These studies have tended to use different criteria; for example, some researchers select plants according to randomized criteria while others use a pharmacological or therapeutic criterion. These latter experiments permit a scientific evaluation of the medicinal use of plants by assessing their pharmacological and toxicological effects. Some studies even shed light on the mode of action of the compounds present in a given plant. Still other researchers study the more representative compounds present in the active extract, which often leads to further studies of plants with related phytochemicals. Research on phenolics from green tea and cocoa, for instance, established the antioxidant properties of these compounds along with their consequent

vascular effects, such as the inhibition of endothelial cell-mediated low-density lipoprotein (LDL)-cholesterol oxidation, hepatoprotection and other related effects (Keen et al., 2005). In parallel, other groups have studied different medicinal plants containing phenolics as potential antioxidants in order to find new sources of active compounds for preventing pathologies associated with oxidative stress, and, in some cases, for their possible use as pharmaceuticals or nutraceuticals.

Future research on the activity of plants—whether known or new—for use in human healthcare requires the establishment of a series of characteristics. The use of standardized and normalized plant extracts, for example, will be of the utmost relevance for evaluating the quality of the samples since one of the major obstacles to using plants as pharmaceuticals is the lack of reproducibility of many published results (Cordell, 2000). In the case of *Ginkgo biloba* L., the use of standardized extracts has allowed researchers to run various studies in different countries with the same extract, thereby permitting comparisons between the results obtained for each research group in order to reach solid conclusions about the pharmacology and toxicology of the plant extract. Also, while research on the active compounds and their mechanisms of action are welcome, the aim of this research should be to shed light on the activity of the extract as a whole; thus, studies should be developed based on the total extract or active purified fraction. This is especially important because the activity of medicinal plants is often the result of the additive or synergistic effects of all their components. For this reason, new techniques such as high-throughput drug studies, which are of great importance for discovering the activity of single compounds, are not always effective for studying the pharmacological activity of medicinal plant extracts (Raskin et al., 2002).

In some cases, clinical studies and the epidemiologic data obtained from people treated with a given medicinal plant can also reveal any damage it may produce. Obviously, this type of study is of immense interest for the future use of plants and crude extracts in medicine. In the case of comfrey (*Symphytum officinale* L.), for example, studies have shown that this plant causes liver damage to patients. In fact, it causes veno-occlusive disease, a non-thrombotic obliteration of the small hepatic veins which leads to cirrhosis and eventually liver failure, sometimes inducing acute or chronic clinical signs with portal hypertension, hepatomegaly and abdominal pain as the main features. This undesired effect with all its toxic signs makes comfrey unsuitable for therapeutic use (Stickel and Seitz, 2000).

Medicinal plants and clinical trials

Although pharmacological experiments are basic for a better understanding of medicinal plants, the development of clinical trials with standardized

extracts is essential for evaluating their potential use in medicine. In fact, this is the usual way of establishing the efficacy and clinical safety of a plant extract. The present progress of phytotherapy in the Western world is essentially due to recent studies that confirm the efficacy and safety of many medicinal plants against certain pathologies. For example, the aforementioned *Ginkgo biloba* has been widely studied in clinical trials that have demonstrated its effectiveness in treating cerebral insufficiency and peripheral occlusive disease. Indeed, it was found to be basically as effective as standard drugs in clinical studies of dementia and was more effective than a placebo. In other neurosensory disturbances such as tinnitus, however, its effect was not significantly different from that of a placebo (ESCOP, 2003). A meta-analysis of ginkgo for intermittent claudication assessed eight double-blind, randomized clinical trials and suggested a significant, but modest increase in pain-free walking distance when compared with a placebo. Another systematic review identified nine double-blind, placebo-controlled, randomized clinical trials of ginkgo for symptomatic treatment of dementia and suggested that the substance is effective in delaying the clinical deterioration of patients and in bringing about symptomatic improvement. Moreover, in this review ginkgo was found to be an effective treatment against tinnitus (Ernst, 2005). Other clinical trials demonstrated the efficacy of garlic against hypercholesterolaemia and hypertension, but the efficacy of ginseng for physical and psychomotor performance, cognitive function, immunomodulation, diabetes and herpes simplex has not been demonstrated and remains uncertain (Ernst, 2005).

Another relevant plant extract is the standardized hydroalcoholic extract of St. John's wort (*Hypericum perforatum* L.), which has been subjected to over 30 clinical studies involving more than 15,000 patients. It has been demonstrated to be effective against mild to moderate, but not severe depression. The studies were performed with both a placebo and a standard antidepressant as a control (ESCOP, 2003).

The Present and Future of Medicinal Plants

Thus, while many plants are used in folk medicine against different pathologies, questions remain. Are they really effective? What kind of pathologies are clearly modified by the use of medicinal plants?. Are plants effective against cancer or acquired immune deficiency syndrome (AIDS)? Some of these questions are answered in this section, in which we review studies on the effectiveness of medicinal plants and their extracts against different pathologies such as infectious diseases, cancer, inflammatory processes or metabolic pathologies. From Table 1, which lists

Table 1. Comparison of number of articles on some relevant pathologies published from 1969 to 1999 and from January 2000 to April 2006 *Source:* PubMed.

Medicinal plants &	*1960-1999 (April)*		*2000-2006 (April)*	
	General	*Reviews*	*General*	*Reviews*
Anti-inflammatory activity	694	47	464	76
Antimicrobial activity	247	12	208	8
Cancer	1767	162	847	111
Metabolism	8748	437	3269	218

Table 2. Experimental and clinical studies on some relevant medicinal plants published from 1969 to 2006 (April) *Source:* PubMed.

Medicinal plant		*Experimental research*		*Clinical trials*	
English name	*Scientific name*	*General*	*Reviews*	*General*	*Reviews*
Aloe	*Aloe vera*	770	59	44	17
Arnica	*Arnica montana*	173	16	27	5
Calendula	*Calendula officinalis*	144	3	9	0
Cranberry	*Vaccinium macrocarpon*	329	48	51	13
Garlic	*Allium sativum*	2464	301	196	52
Ginkgo	*Ginkgo biloba*	1682	233	352	79
Ginseng	*Panax ginseng*	2530	206	169	38
Devil's claw	*Harpagophytum procumbens*	76	9	16	5
St John's wort	*Hypericum perforatum*	1142	173	216	55
Licorice	*Glycyrrhiza glabra*	1697	152	122	10
Saw palmetto	*Serenoa repens*	221	58	87	27
Senna	*Cassia senna* and *C. angustifolia*	489	27	64	5
Valerian	*Valeriana officinalis*	465	52	64	15

various publications concerning the action of medicinal plants on a selected number of diseases, one can note a marked increase in publications from 2000 to 2006 as compared to the period between 1960 and 1999.

Some medicinal plants, those which are the most representative in phytotherapy, are the repeated subject of various experimental and clinical studies. Table 2 shows the number of papers on these representative species and the numerous clinical trials that have been carried out with them during the past 37 years.

Medicinal plants as anticancer agents

The general term cancer includes a series of diseases in which the body's cells become abnormal and divide without control. Cancer cells may invade nearby tissues and spread through the bloodstream and lymphatic system to other parts of the body. Medicinal plants are often mentioned in different studies as potential anticancer agents. They are obviously not a cure for the disease, but they may provide a good source from which to obtain active compounds. Thus, of the at least 250,000 species of plants on the earth, more than 1000 have shown significant anticancer properties (Cordell et al., 1993). While many molecules obtained from nature have shown great progress, the chemistry of numerous molecules remains to be studied (Mukherjee et al., 2001). For this reason, preclinical *in vitro* studies to select potential anticancer agents are welcome. Several classic cases of compounds obtained after a selective study of cytotoxicity include vinblastine and camptothecin (alkaloids), podophyllotoxin and rabdosiin (lignans), paclitaxel and cumindysosides (terpenoids), punicalagin and sanguiin (polyphenolics). Still, these are only some of the more representative antineoplastic agents that have been obtained from medicinal plants (Lee, 1999).

The first step in obtaining potential antineoplastic agents is the selection of a good general method of study. An adequate method for studying both the cytotoxicity and the correct dilution of the extract allows researchers to identify specific active extracts which can then be studied in more depth in secondary studies with the aid of more specific methods. The second consideration is the selection of adequate plants. A classic case of a fruitful study was that of *Podophyllum peltatum* L., which was described in the literature as potential anticancer plant. The isolation of its bioactive compound, podophyllotoxin, led to the subsequent synthesis of related compounds such as etoposide and teniposide.

Developing new drugs from active compounds is no easy task as the supply of such compounds may be limited not only by the quantity of active compounds in a plant, but also by the plant's growth rate, the limited localization of active ingredients in the specific organs, and the desire to conserve natural resources (Tabata, 2006). These problems affected the ease with which paclitaxel, for example, was obtained. This cytotoxic compound was originally isolated from *Taxus brevifolia* L. in extremely scant amounts, but using synthetic techniques, researchers were able to obtain the compound from the more readily available 10-deacetyl-baccatin III, a compound isolated from *Taxus baccata* L., in much higher yields (Lee, 1999; Mukherjee et al., 2001).

Detection of new metabolites with antineoplastic activity is one of the principal aims of medicinal plant research. A review of studies with this

focus shows that in the last 5 years about 850 research papers have been published on this subject, 111 of which were reviews. However, many are studies on cytotoxicity against selected cells and the activity is not well defined. This fact underscores the need for a careful choice of method, extract concentration and product to be tested. Thus, for studying the activity of a medicinal plant extract, the initial concentration should be adequately established and the protocol to determine potential active principles should be clearly defined. Another problem is that while *in vitro* screening provides useful information on the cytotoxicity and relative potency of an extract or isolated compound, the activity does not always carry over *in vivo* (McLaughlin, 1991). The problem is that *in vivo* bioassays are comparatively more expensive, time-consuming and complicated. In addition, they are sometimes not reproducible and may be statistically unreliable. They are also extract-depleting, which makes them unsuitable for the screening of antitumor activity (McLaughlin, 1991).

Do medicinal plants thus have potential as anticancer agents? Yes, but only as a source of active compounds. In this vein, natural products are an extremely interesting source of antineoplastic agents. One example is camptothecin, isolated from *Camptotheca acuminata* Decaisne, and its derivatives (topotecan, irinotecan and others), which are potent antitumor and DNA topo I inhibitory agents. Another interesting compound is podophyllotoxin from *Podophyllum peltatum*, which inhibits mitosis by reversibly binding to tubulin and inhibiting microtubule assembly while its analogue inhibits the essential enzyme DNA topo II, thereby increasing DNA cleavage. Other compounds that have been isolated from medicinal plants are vinblastine and vincristine from *Catharanthus roseus* (L.) G. Don, which are major drugs in the treatment of Hodgkin's lymphoma and acute childhood leukemia, respectively. Their synthetic derivatives have led to various analogues such as vindesine, which shows activity against other tumors, exhibits less toxicity and has fewer side effects. Other compounds with only one mode of action include the aforementioned paclitaxel, a mitotic inhibitor that promotes the assembly of microtubules. This compound has been used selectively to treat breast and ovarian cancer (Lee, 1999; Mukherjee et al., 2001).

Recently, Kimura (2005) reviewed the antitumoral and antimetastatic effects of various natural products, describing the properties of stilbene derivatives such as resveratrol and cassiagrol A (stilbene dimer), which are isolated from *Polygonum* and *Cassia* species. These derivatives were found to display both effects in different *in vitro* and *in vivo* models through the inhibition of tumor-induced neovascularization. Moreover, two chalcone derivatives from *Angelica keiskei* Koidzumi also inhibited tumor growth and metastasis in tumor-bearing mice through the inhibition of

tumor-induced neovascularization and/or the inhibition of immune suppression caused by tumors (Kimura, 2005). Of all the natural products, however, the sesquiterpene lactones are one of the groups with the most potential as anticancer drugs. Several different studies on these lactones have established a correlation between the *in vitro* effects and the structure-activity relationship. The active compounds all have a common functional structure, an α-methylene-γ-lactone group, and they act on cell signalling pathways such as nuclear transcription factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK). They may thus be promising anticancer agents with potential applications in both cancer chemotherapy and chemoprevention (Zhang et al., 2005).

Chemoprevention, especially against skin cancer is another possible application for medicinal plants as anticancer agents. Ultraviolet radiation from the sun is not only responsible for the majority of skin damage, but also for both the initiation and the promotion of the various phases of skin cancer. Taking this into account, photochemoprevention by means of natural products could ameliorate the adverse effects of ultraviolet radiation on the skin. Of special interest are the natural antioxidants obtained from green tea (*Camellia sinensis*), turmeric (*Curcuma longa* L.), milk thistle (*Silybum marianum* (L.) Gaertner), garlic (*Allium sativum* L.), ginkgo (*Ginkgo biloba*), red clover (*Trifolium pratense* L.) and soy (*Glycine max* (L.) Merrill). The antioxidant compounds that have been obtained from these plants include epigallocatechin-3-gallate, epigallocatechin and epicatechin gallate from tea; curcumin from turmeric; silymarin from milk thistle; genistein from soy, red clover and ginkgo; and resveratrol, apigenin, carotenoids, α-tocopherol and caffeic and ferulic acids from various sources. All of these isolated compounds prevent ultraviolet-mediated carcinogenesis through different mechanisms, but in general, when applied as sunscreens, they may reduce ultraviolet-generated, radical oxygen species-mediated photodamage, immunosuppression and skin cancer in humans (F'guyer et al., 2003). The possible use of medicinal plants and their products in radioprotection was reviewed by Arora et al. (2005), who compiled a series of species used in Ayurveda to treat free radical-mediated ailments. They selected *Centella asiatica* L., *Ginkgo biloba*, *Hippophae rhamnoides* L., *Mentha piperita* L., *Ocimum sanctum* L., *Panax ginseng* C.A. Meyer, *Podophyllum hexandrum* Royle and *Tinospora cordifolia* Miers ex Hook. f. & Thomson, which were found to exhibit an array of biological activities that could be relevant for mitigating ionizing radiation-induced damage in mammalian systems. However, no clinical trials have been developed to establish their effect on human health.

Medicinal plants with anti-inflammatory activity

Different plant extracts have been subjected to specific studies to establish their efficacy and toxicity in complementary and alternative medical therapies for rheumatic conditions. Of these, *Harpagophytum procumbens* L. may have an application in the treatment of low back pain while *Tripterygium wilfordii* Hook. f. and *Uncaria tomentosa* (Willd) DC seem to be interesting for the treatment of rheumatoid arthritis and *Urtica dioica* L. and *Salix alba* L. are effective against osteoarthritis. However, *Tanacetum parthenium* L., which inhibited the expression of the intercellular adhesion molecule (ICAM)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IκB kinase and also decreased T cell adhesion, had no demonstrable effect in the treatment of rheumatoid arthritis in human studies (Setty and Sigal, 2005).

Some medicinal plants contain compounds with specific activity against pro-inflammatory enzymes such as cyclooxygenase (COX), lipoxygenase (LOX) and nitric oxide synthase (NOS). Indeed, several of these plants served as a springboard for the synthesis of new compounds with higher activity, such as salicylic acid. Recent studies have demonstrated the mechanism of action of many known medicinal plants, opening the way to possible new heads of series for novel anti-inflammatory drugs. One example is parthenolide, obtained on the basis of feverfew (*Tanacetum parthenium*), which inhibits the expression of COX-2, thereby preventing prostaglandin (PG) generation. Another example is yomogin from *Artemisia princeps* Pamp., which inhibits nitric oxide (NO) production by suppressing NOS expression (Sautebin, 2000). Flavonoids are another source of interesting active compounds for the treatment of inflammatory diseases. Some of these compounds, such as citrus flavonoids, inhibit TNF-α release (Habtemariam, 2000) while others inhibit the various lipoxygenases. Thus, while baicalein specifically inhibits 5-LOX, cirsiliol is a specific inhibitor of 12-LOX (Middleton et al., 2000) and quercetin inhibits 15-LOX (Sadik et al., 2003). Such compounds could serve as new and specific models for obtaining selective inhibitors of inflammatory diseases and other pathologies such as thrombosis, osteoporosis, cancer and viral infections (Nijveldt et al., 2001).

In 2004, Gagnier et al. reviewed the effect of devil's claw (*Harpagophytum procumbens*) on osteoarthritis and low back pain and concluded that there is limited evidence for the efficacy of the plant's ethanol extract at doses containing less than 30 mg harpagoside per day in the treatment of knee and hip osteoarthritis. Moderate evidence exists of the effectiveness of the use of root powder with 60 mg of harpagoside in the treatment of osteoarthritis of the spine, hip and knee as well as for the use of an aqueous extract at a daily dose of 100 mg of harpagoside in the treatment of acute

exacerbations of chronic, non-specific low back pain and for the use of an aqueous extract at 60 mg of harpagoside for short-term treatments of chronic, non-specific low back pain. Finally, there is strong evidence that the use of an aqueous extract at a daily dose equivalent to 50 mg of harpagoside is effective in the treatment of acute exacerbation of chronic, non-specific low back pain. Review of systematic trials concluded that the efficacy of devil's claw products is not transferable from product to product, and that extracts containing at least 50 mg harpagoside in the daily dosage were more active than products containing less, as was the case with the ethanolic extracts (Chrubasik et al., 2003).

Recently, two high quality trials examined the effects of devil's claw in the treatment of low back pain and found strong evidence that daily doses standardized to 50 or 100 mg of harpagoside were better than a placebo or rescue medication for short-term improvements in pain. Another high quality trial demonstrated a relative equivalence between devil's claw and 12.5 mg per day of the selective COX-2 inhibitor rofecoxib (Gagnier et al., 2006). Although experimental studies *in vitro* seem to indicate that *Harpagophytum procumbens* inhibits eicosanoid biosynthesis (Setty and Sigal, 2005), clinical studies in healthy human volunteers indicated that there was no significant effect on eicosanoid mediators such as PGE_2, tromboxane B_2 (TXB_2), 6-ketoPGF$_1\alpha$ and leukotriene B_4 (LTB_4) after three weeks of a daily treatment with 4 × 500 mg of powdered roots standardized with 3% iridoids. The study concluded that *Harpagophytum procumbens* did not act by a mechanism characteristic of anti-arthritic drugs of the non-steroidal, anti-inflammatory type. In addition, although the active principle of *Harpagophytum procumbens* has yet to be identified, harpagoside is used as a marker for standardization (Gagnier et al., 2004).

Trials examining the effects of *Salix alba* on low back pain found moderate evidence that daily doses standardized to 120 mg or 240 mg salicin were better than a placebo or rescue medication for short-term improvements in pain, with a relative equivalence to 12.5 mg per day of rofecoxib (Gagnier et al., 2006).

Aloe vera L. is one of the best-known herbal remedies for the treatment of different diseases, especially skin diseases, but in a double-blind, placebo-controlled study of a commercial gel in the treatment of slight to moderate psoriasis vulgaris, only a modest effect was obtained; indeed, it was no better than the placebo effect. However, the high response rate for the placebo indicates a possible effect for this in its own right, which thus reduces the observed therapeutic effect by comparison (Paulsen et al., 2005). These types of results are frequent, perhaps because in some cases the placebo may actually contain substances with effects similar to those of the pharmacological agent used in the experiment. In this particular case,

a weak hydrating effect could be enough to cause a good to medium response against the inflammatory pathology. Other specific studies have clearly demonstrated the positive effects of polyunsaturated fatty acids and antioxidants on psoriasis when administered in the diet (Wolters, 2005). Some fatty acid derivatives such as eicosapentaenoic acid (C20:5n3) from fish (mackerel and herring) act as a competitive inhibitor of arachidonic acid conversion to PGE_2 and LTB_4. Since high levels of arachidonic acid and LTB_4 have been measured in the skin and erythrocyte membranes of patients with psoriasis, eicosapentaenoic acid has been found to reduce the inflammation in psoriatic skin by means of a competitive mechanism with arachidonic acid in its metabolization to LTB_4. In addition, the acid seems to raise superoxide anion liberation in psoriatic dermal fibroblasts, a process which is implicated in the inflammatory mechanism of psoriasis. Patients with psoriasis exhibit several markers of oxidative stress and a sufficient amount of antioxidants may thus be helpful in preventing an imbalance of oxidative stress and antioxidant defence in psoriasis (Wolters, 2005).

Plants with antimicrobial and antiviral activities

In the past few decades, the search for new anti-infectious agents has occupied many research groups in the field of ethnopharmacology. Recently, Ríos and Recio (2005) reviewed the number of articles published on the antimicrobial activity of medicinal plants in PubMed during the period between 1966 and 2004 and found 422 articles. In these studies, the antimicrobial activity of plant extracts was attributable to different types of chemical compounds, including alkaloids, flavonoids, sesquiterpene lactones, diterpenes, triterpenes or naphtoquinones, among others. Some of the plants are extensively used not only in folk medicine, but also in phytotherapy. Some examples include the use of bearberry (*Arctostaphylos uva-ursi* L.) and cranberry juice (*Vaccinium macrocarpon* Ait.) to treat urinary tract infections, the use of lemon balm (*Melissa officinalis* L.), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia* (Maiden et Betch) Cheel) as broad-spectrum antimicrobial agents and the use of the latter for treating acne and infectious skin problems (Ríos and Recio, 2005).

Use of essential oils to treat different diseases constitutes a widely used system of natural product therapy. Aromatherapy, for instance, is the use of concentrated essential oils extracted from herbs, flowers and other parts of plants to treat diseases ranging from skin infections to anxiety (Cooke and Ernst, 2000). Among the various uses of such oils, the principal one is in relation to different types of respiratory, urinary tract and skin infections. Essential oils obtained from frankincense, petitgrain, bergamot, sweet orange, lemongrass, eucalyptus, jasmine, juniper, lavender, tea tree,

cajeput, niaouli, melissa, peppermint, basil, geranium pine, pepper, patchouli, rose, rosemary, savoury and benzoin all have a relevance in dermatology. However, the number of controlled clinical trials has been small (Stevensen, 1998). Of all the oils tested, tea tree oil is the most thoroughly and best studied from a clinical point of view. Indeed, its properties against onychomycosis have been clearly demonstrated, along with its effects against different bacteria. However, several grave cutaneous side effects after treatment with this essential oil have also been observed (Mevorah et al., 2003).

The activity of medicinal plant extracts against viruses *in vitro* has been widely assayed by various research groups. However, the results of these studies must be taken with a pinch of salt, as *in vitro* studies are often invalid in the case of viral infections. For example, from Jassim and Naji's (2003) review of screening studies of medicinal plants against various viruses it became clear that compounds requiring concentrations of 250 µg/ml to be effective cannot be considered as future therapeutic agents. Nevertheless, the data from these studies are useful as a springboard for the synthesis of new antiviral agents through structural modification of the active compounds isolated from extracts. Indeed, there is a wide spectrum of chemical compounds which exhibit antiviral activity, including coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtoquinones, anthraquinones, polysaccharides, proteins and peptides (Jassim and Naji, 2003).

Several parameters must be set when studying antiviral activity as different results are often described for different methods of extraction, for example. Thus, different studies have examined the aqueous extracts of plants such as *Acacia nilotica* L. and *Maytenus senegalensis* (Lam.) Exell, the methanol extracts of species such as *Dodonaea angustifolia* L. and the acetone extracts in the case of *Combretum paniculatum* Vent. (Jassim and Naji, 2003). There are also clear differences depending on the season of collection. For instance, *Phytolacca americana* L. gives three different pokeweed antiviral proteins (PAP) depending on the season in which the leaves are collected; thus, spring, early summer and late summer give PAP-I, PAP-II and PAP-III, respectively. These proteins cause concentration dependent depurination of genomic human immunodeficiency virus (HIV)-1 RNA (Rajamohan et al., 1999).

Many contradictory results have been obtained in studies on *Phyllanthus amarus* Schumach. & Thonn., a medicinal plant used in India as an antiviral agent. Different trials have demonstrated that standardized extracts containing 20 mg of geraniin have no effect on the levels of hepatitis B surface antigen (HBsAg) and that the plant powder does not reduce the duration of jaundice in hepatitis B virus (HBV) patients.

However, *in vitro*, the *Phyllanthus amarus* extracts inhibited the secretion of HBsAg and suppressed mRNA transcription of the HBV by means of a specific mechanism of action (Jassim and Naji, 2003). These results justify the necessity of running clinical trials to confirm this activity *in vivo.*

With respect to the efficacy of such extracts as antiviral agents, several interesting clinical trials have been published all ratifying their use in folk medicine and confirming the potential use of plant extracts against viral infections. Of the studies reviewed, the most relevant involves the study of five patients with chronic hepatitis C who were treated for one year with a standardized extract of *Viscum album* L. The treatment reduced hepatitis C virus (HCV) production by 6 to 20-fold in two of the patients, thus normalizing their liver functions and improving their quality of life without side effects (Tusenius et al., 2001).

In a similar fashion, andrographolide from *Andrograhis paniculata* (Burm. f.) Wall ex Nees was studied in a clinical trial as a potential anti-HIV agent on 13 HIV positive patients and 5 HIV negative healthy volunteers. At the end of the trial there was a significant increase in the CD4+ lymphocyte level of the HIV-infected patients (Calabrese et al., 2000).

A double-blind, placebo-controlled, randomized trial on 66 patients with recurrent herpes simplex virus (HSV) infections was performed with a cream containing a standardized leaf extract of *Melissa officinalis*. The results demonstrated the activity of the extract against herpes simplex labialis without cytotoxic side reactions (Koytchev et al., 1999), but new studies on herpes simplex infections of the genital mucosa and HSV-2, which invades the sciatic nerve ganglia, could be of interest for future trials (Jassim and Naji, 2003).

Coneflower (*Echinaceae* species) has also been used for the treatment of genital herpes. Although some pharmaceutical products containing *Echinacea purpurea* (L.) Moench extract have been reported to be antiviral agents against HSV-1 and HSV-2, there is insufficient clinical evidence to support the use of this medicinal plant in the treatment of herpes (Perfect et al., 2005). Other medicinal plants such as *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. and *Aloe vera*, have also been described as active against the herpes virus; however, while the first may be useful in reducing the severity, duration and frequency of outbreaks, the duration of treatment seems excessive compared with that of the standard treatment. It is thus only useful for suppressive rather than episodic therapy. The clinical studies realized with the latter species showed a reduction of symptom resolution as compared to the placebo effect; however, these studies were only performed on men. Without similar studies on genital herpes among women, the efficacy of this plant cannot be clearly and completely established (Perfect et al., 2005).

In 2003, Kalemba and Kunicka reviewed antimicrobial properties against some bacteria and fungi of essential oils and their constituents and cited more than 500 reports. The essential oils from thyme (*Thymus vulgaris* L.), origanum (*Origanum vulgare* L.), mint (*Mentha piperita*), cinnamon (*Cinnamomum cassia* Blume), salvia (*Salvia officinalis* L.) and clove (*Syzygium aromaticum* (L.) Merrill et L.M. Perry) were found to possess the strongest antimicrobial properties among many tested. One year later, Martin and Ernst (2004) published a systematic review on antifungal herbal preparations that were tested in controlled clinical trials. Of them, tea tree oil preparations were tested in four randomized clinical trials and some positive outcomes were attributed to the intervention in all trials. In addition, two trials of *Solanum* species and one trial oil of bitter orange preparations were compared with conventional treatments. In all cases encouraging results were reported, but the authors conclude that there are few controlled clinical trials of herbal antifungal medicines, and only tea tree oil present some promise and solicit further investigations in rigorous clinical trials.

Medicinal plants and digestion

Phytotherapy for the digestive apparatus is quite popular in Germany and France, where medicinal plants are widely used for treating digestion troubles, chronic constipation and diarrhoea. In many cases the symptoms improve with dietary measures alone, but in some cases an adjuvant treatment may be of interest. In the case of diarrhoea, the inclusion of starch from rice, maize or potatoes in the diet serves as a rehydration measure while for constipation the use of laxatives with a different mechanism, e.g. a bulk-forming agent or a stimulant laxative, could be of interest. Examples of the former include ispaghula (*Plantago ovata* Forssk.), psyllium (*Plantago psyllium* L.) and flaxseed (*Linum usitatissimum* L.), which all have a high percentage of fibre and polysaccharides that swell in the gastrointestinal tract. In the case of the latter, plants with anthraquinone-derivatives are known to increase peristalsis in the colon, reducing both transit time and the reabsorption of water from the colon. These kinds of principles, however, tend to produce colic and gripping pains due to the spastic contractions of the smooth muscle (Heinrich et al., 2004). Plants containing these principles include senna (*Cassia senna* L. and *C. angustifolia* Vahl), frangula (*Rhamnus frangula* L.), cascara (*R. purshiana* DC) and rhubarb (*Rheum palmatum* L.).

The treatment of chronic constipation comprises one of the most used—and abused—applications of medicinal plants. Recently, Ramkumar and Rao (2005) reviewed the efficacy and safety of traditional medical therapies for chronic constipation and found that there is little evidence to justify

the use of stimulant laxatives such as Senna and other anthraquinones. The studies that exist are limited in number and power and flawed in their design and/or conduct, all of which reduces their validity and safety. Moreover, there is little evidence to support a recommendation for or against the use of the modality itself. In the case of psyllium or ispaghula, their seeds do seem to improve stool frequency, but different conclusions were reached, with some clinical trials suggesting that total gut transit improved while others observed no difference with respect to a placebo. In summary, the reviewers concluded that the use of ispaghula for chronic constipation is supported by a fair level of evidence because most of the results show some kind of benefit, but the strength of this conclusion is limited by the number, quality and consistency of the individual studies. Their recommendation for using this medicinal plant in the treatment of constipation was therefore only moderate.

Milk thistle (*Silybum marianum*) is used to treat viral infections, cirrhosis of the liver and to protect the liver against toxins. Diverse clinical trials have shown conflicting evidence of its efficacy; the authors thus recommend that patients should be informed that while there is insufficient evidence to prove the effectiveness of milk thistle in the treatment of viral hepatitis and alcoholic liver disease, at least it appears to be safe. The evidence for cytoprotection is likewise limited, but the available data suggest some benefits. As for the extract's anticarcinogenic properties, clinical trials are currently under way (Rainone, 2005).

Licorice (*Glycyrrhiza glabra* L.) has been widely used in Europe at least since the 4th century BC. It was used even earlier in ancient Assyria, Egypt, China and India. Its use against diseases of the respiratory, gastrointestinal, cardiovascular and urogenital systems as well as against various skin diseases is widely documented from the time of the ancient Greeks to the present day. The comparison of its medical uses in different cultures has demonstrated the validity of specific indications in different ethnic groups with different cultural backgrounds (Fiore et al., 2005). However, some of these properties, such as its use in gastric ulcer disease, have yet to be proven in clinical trials. In addition, other newly discovered properties of licorice should be included in future clinical research, including its antiviral effects against different respiratory viruses, its effect on allergic/inflammatory skin diseases and its anti-atherogenic properties. However, all studies should take into account the mineralocorticoid properties of this extract. These arise from its inhibition of cortisol metabolism and constitute the principal non-desirable effect of licorice, as they may increase sodium and decrease potassium levels as well as cause water retention, all of which could affect the arterial tension in many patients with hypertension (Fiore et al., 2005). Licorice is thus not a totally innocuous substance and its use should be carefully controlled.

Medicinal plants and metabolism

Changes in the metabolism of lipids or glucose causes metabolic alterations, many of which give rise to diseases such as atherosclerosis or diabetes. In the case of the latter, one specific type, namely diabetes mellitus, is a metabolic disorder of the endocrine system which is common throughout the world. Of the various kinds of diabetes, type 2 or noninsulin-dependent diabetes mellitus could, in part, be treated with medicinal plants. Still, while there are many references to studies of this type, not enough clinical trials have been reported in the literature. Li et al. (2004) reviewed the natural medicines traditionally used in China for the therapy of diabetes mellitus while Mukherjee et al. (2006) reviewed the medicinal plants with hypoglycaemic potentials used in traditional Indian medicine. In the former, the authors compiled a list of the 29 medicines most frequently used in anti-diabetic compound recipes, along with another 22 traditional Chinese medicines often used in anti-diabetic compound recipes, 13 traditional Chinese medicines or natural products commonly used in diets to control and/or treat diabetes and its complications and 18 medicinal plants with outstanding anti-diabetic potential (Li et al., 2004). Claims for the effects of these plants and plant extracts are based on their traditional use and on experimental pharmacology data from studies with animals, but there is no data from clinical studies in humans. In the review carried out by Mukherjee et al. (2006), 65 species and 7 phytoconstituents with hypoglycaemic potential are included. Some are currently being investigated in clinical trials, but there are no specific references to these studies.

Plant sterols and stanols (saturated sterols) are a potential alternative for lowering plasma cholesterol levels as they have been found to reduce plasma total- and LDL-cholesterol. This is probably due to the fact that, despite their similar chemical structures, plant sterols have poor intestinal absorption as compared to cholesterol. Other biological activities of phytosterols, including their effects on lecithin:cholesterol acyltransferase activity, bile acid synthesis, oxidation and uptake of lipoproteins, hepatic and lipoprotein lipase activities and the coagulation system, have all been linked to their anti-atherogenic properties (Moghadasian, 2000).

Silymarin, a mixture of flavonolignans from milk thistle (*Silybum marianum*), is used in the treatment of liver diseases because of its hepatoprotective activity, which is considered to involve antioxidative and membrane stabilizing effects. Liver injury is often reflected as a secondary hyperlipoproteinemia, which may lead to development of atherosclerosis, particularly when associated with hypercholesterolaemia. It is thought that silymarin may be of some benefit with regard to liver lipid metabolism and the regulation of plasma lipoproteins (Skottova and Krecman, 1998).

Panax ginseng has been described as a stimulant of both carbohydrate and lipid metabolism. The reports, however, are often contradictory, perhaps due to differences in methods of extraction, subsequent treatments, or even the season of collection (Gillis, 1997). In addition, some of the active principles, namely the panaxans, have been identified and found to elicit hypoglycaemia in both normal and diabetic mice. In addition, adenosine, a carboxylic acid and a peptide were all found to inhibit catecholamine-induced lipolysis in rat epididymal fat pads (Ng and Yeung, 1985). However, clinical trials did not corroborate these results.

Garlic (*Allium sativum*) exhibits hypolipidemic, antiplatelet and procirculatory effects. Although not all of garlic's active ingredients are known and allicin-like transient components are not directly active, ample research suggests that an allicin-free garlic preparation standardized with a bioavailable component such as *S*-allylcysteine is active and that various effects of garlic may be attributed to it. Furthermore, various chemical constituents in garlic products, including nonsulphur compounds such as saponins, may contribute to the essential biological activities of the plant (Amagase, 2006). Several garlic compounds can suppress LDL oxidation *in vitro*. This is important because when oxidized (not in its native state), LDL promotes vascular dysfunction by several means, namely by exerting direct cytotoxicity toward endothelial cells, by increasing chemotactic properties for monocytes, by transforming macrophages to foam cells via scavenger-receptors and by enhancing the proliferation of various cell types. Indeed, short-term garlic supplementation in human subjects was shown to cause an increased resistance of LDL to oxidation and a consequent reduction of both atherogenesis and risk for cardiovascular diseases (Lau, 2001).

In a randomized, double-blind, placebo-controlled intervention study, we showed that aged garlic extract supplementation was effective in lowering plasma concentration of total cholesterol by 7% and LDL cholesterol by 10% in hypercholesterolaemic men compared with subjects taking a placebo. Further experimental studies have suggested that hydrophilic and hydrophobic compounds of garlic actually inhibit cholesterol synthesis (Yeh and Liu, 2001). However, on the basis of numerous rigorously designed controlled studies, there is little unequivocal evidence for the lipid-lowering properties of garlic preparations (Berthold and Sudhop, 1998; Spigelski and Jones, 2001).

Medicinal plants in the treatment of heart disease, hypertension and vascular diseases

Crude drugs used in diseases of the cardiovascular system are of great relevance in phytotherapy. Prevention of hypercholesterolaemia with the

aid of plant extracts could prevent atherosclerosis while the use of medicinal plants is a good complement to diet in the prevention and treatment of hypertension. In fact, within the field of phytotherapy, the number of studies on medicinal plants that exert activity on the cardiovascular system is quite significant, as can be seen in Table 3.

Table 3. Experimental researches on some relevant cardiovascular pathologies published from 1969 to 2006 (April) *Source:* PubMed.

Medicinal plants &	*General*	*Reviews*
Arteriosclerosis	161	20
Heart disease	1522	144
Hypertension	557	83
Vascular diseases	1394	142

The main cause of heart and vascular diseases is atherosclerosis and its associated risk factors. These include hypertension and hypercholesterolemia, which can activate several pro-inflammatory enzyme systems including those of xanthine oxidase, NADH/NADPH oxidase, and myeloperoxidase. Upon activation, these systems produce reactive oxygen species that can have an effect on NO and LDL, as well as on the endothelia. Polyphenols from cocoa (flavanols and procyanidins) can stimulate NO production and reduce xanthine oxidase and myeloperoxidase activity, but they also suppress the production of pro-inflammatory cytokines and 15-LOX activity, enhance the production of anti-inflammatory cytokines, and positively modulate the effect of transforming growth factor (TGF)-β and TNF-α. These mechanisms are in part modulated by nuclear factor-κB (NF-κB) gene transcription, which is itself modified by the antioxidant activity of polyphenols. This is due to the fact that NF-κB is a transcription factor sensitive to oxidant stimuli and to changes in the cellular thiol redox state (Keen et al., 2005).

Antioxidants from plants may also affect numerous intracellular signalling cascades and influence the cardiovascular system by enhancing vascular function and decreasing platelet activity. Some foods and plant extracts provide a high concentration of compounds with antioxidant capacity; these include tea, red wine, blueberries, garlic, strawberries and cocoa (Keen et al., 2005). Green tea (*Camellia sinensis* (L.) Kuntze) contains a high level of polyphenols and as such was found to improve endothelium-dependent, flow-mediated dilatation of the brachial artery in patients with coronary artery disease (Achike and Kwan, 2003).

Flavonoids are also a source of medicinal agents, some of which act against cardiovascular disease. These substances may have an effect on

the contraction of cardiac and vascular smooth muscle cells (Middleton et al., 2000). In general, they have antioxidant effects, which could prevent the endothelial damage induced by oxidative agents by inhibiting lipid peroxidation and its consequent effects (Woodman and Chan, 2004).

The use of hawthorn (*Crataegus oxyancantha* L. syn. *C. laevigata* (Poiret) DC) as an adjuvant in the treatment of heart failure and as an anti-arrhythmic drug is accepted by numerous specialists. Hydroalcoholic preparations of hawthorn (*Crataegus oxyancantha*, and other species) leaves and flowers are recommended for declining cardiac performance corresponding to functional capacity class II as defined by the New York Heart Association (NYHA). This category applies to patients with cardiac disease that causes a slight limitation of physical activity and which may manifest itself in symptoms such as fatigue, palpitations, dyspnoea or anginal pain (ESCOP, 2003). The more relevant compounds found in hawthorn are flavonoids, proanthocyanidins, triterpenes and organic acid (phenylpropanoid derivatives), which confer cardiotonic, anti-arrhythmic, hypotensive, hypolipidemic and antioxidative properties to this plant (Chang and Zuo, 2002). To date, clinical studies seem to confirm the efficacy of this plant in decreasing heart rate, improving tolerance to exercise and pressure/heart rate products and lowering serum lipids in patients with NYHA stage II heart failure or with hyperlipidemia, but no significant effect on diastolic blood pressure has been observed (Chang and Zuo, 2002). The recommended daily dose of hawthorn is 160-900 mg of a native water-ethanol extract of the leaves or flowers, equivalent to 30-169 mg of epicatechin or 3.5-19.8 mg of flavonoids, administered in two or three doses (Rigelsky and Sweet, 2002). An international, multi-centre, prospective clinical study including a large number of NYHA class II and III heart failure patients is currently underway to test hawthorn's long-term therapeutic effects (Chang, 2005).

The use of medicinal plants to treat haemorrhoids and varicose veins is well documented and accepted in traditional and official medicines from both Western and Eastern cultures. Clinical trials have demonstrated that oral supplementation with gotu kola (*Centella asiatica*), horse chestnut (*Aesculus hippocastanum* L.), butcher's broom (*Ruscus aculeatus* L.), witch hazel (*Hamamelis virginiana* L.) or bioflavonoid mixtures may prevent time-consuming, painful and expensive complications of varicose veins and haemorrhoids, with clear subjective and objective improvements in symptoms (MacKay, 2001). In the case of horse chestnut, its most relevant active compound, aescin, has been widely studied and its pharmacological properties demonstrated; the results justify the therapeutic use of this medicinal plant for treating various venous disorders. In fact, a number of different pharmacological experiments and clinical trials have demonstrated the anti-oedematous, anti-inflammatory and venotonic properties of

both aescin and hippocastanus extracts (Sirtori, 2001). The most relevant studies focus on their therapeutic activity and efficacy in chronic venous insufficiency; indeed, of the considerable number of trials that have been carried out with oral horse chestnut extract and aescin, almost all have concluded that these substances are both effective and well-tolerated and that they improve upon the results obtained with flavonoids. In addition, oral horse chestnut extract and aescin have been proven effective against haemorrhoids and post-operative oedema (Sirtori, 2001).

Standardized extracts from ginkgo (*Ginkgo biloba*) leaves are used for acute ischaemic stroke. Zeng et al. (2005) recently reviewed the clinical trials published on this subject and concluded that there was no convincing evidence from trials of sufficient methodological quality to support the routine use of ginkgo extract to promote recovery after stroke. The authors proposed that high-quality, large-scale, randomized, controlled trials are needed to test the efficacy of ginkgo against acute ischaemic stroke.

Medicinal plants as a source of phytoestrogens

Phytoestrogens are a family of plant compounds that act like estrogens in animal cells and bodies. Phytoestrogens are found in different vegetables, including some commonly included in the diet, and they have been correlated with the incidence of many diseases, including coronary heart disease, breast cancer and endometrial and ovarian cancers. They have also been related to menopausal symptoms. From a chemical point of view, phytoestrogens are found in two different structural groups: flavonoids and lignans. The flavonoid group of phytoestrogens includes coumestans, prenylated flavonoids and isoflavones, among others. In the lignan group, only a reduced number of compounds with estrogenic properties have been reported. Vegetables rich in phytoestrogens derived from flavonoids include soybeans and their derivatives (soyflour, tempeh, tofu), while the lignan group comprises flaxseed and its derivatives. Other foods containing these compounds are lentils, seaweed, oat bran, kidney beans, wheat, garlic, squash, asparagus, pears, rye and plums. Most of these are common in the normal diet, but some of them are used as medicinal agents (Knight and Eden, 1996; Mazur and Adlercreutz, 1998; Tham et al., 1998), to obtain enriched extracts for treatment of menopausal symptoms in women. These include different species widely used in phytotherapy such as soy (*Glycine max*); red clover (*Trifolium pratense*) and other *Trifolium* species, which are rich in isoflavones (Figure 1); alfalfa (*Medicago sativa* L.); species of *Brassica* with coumestanes (Figure 2); hops (*Humulus lupulus* L.), which contain prenylflavonoids and chalcones; *Vitis vinifera* L. and some species of conifers with estilbenes (resveratrol) as well as flaxseed and cereals with lignans (Figure 3). In the case of isoflavones, coumestanes and flavonoids,

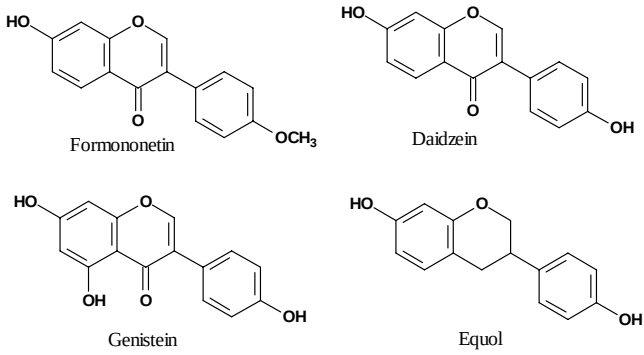


Fig. 1. Phytoestrogens derived from isoflavone structure.

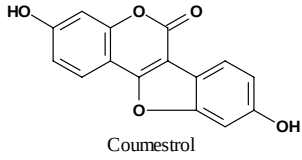


Fig. 2. Phytoestrogens derived from coumestane structure.

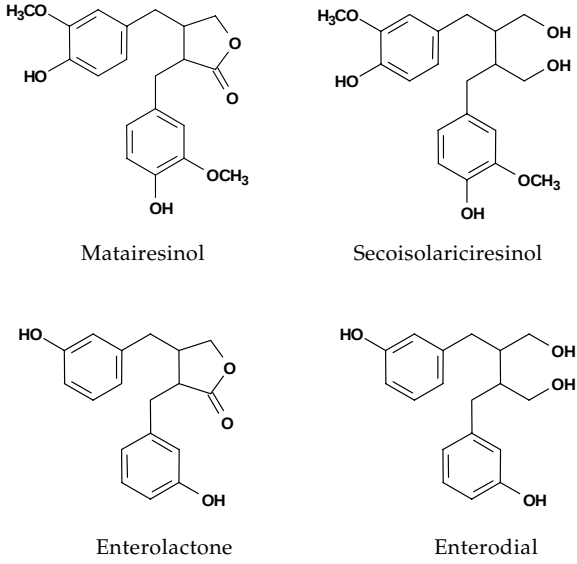
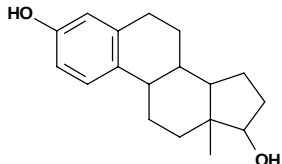


Fig. 3. Phytoestrogens derived from lignan structure.



17β-Estradiol

Fig. 4. Chemical structure of 17β-Estradiol.

the effects are directly mediated by the compounds themselves, which act via estrogen receptors (Tham et al., 1998). However, lignans such as matairesinol and secoisolariciresinol are first metabolized to the active principles enterodiol and enterolactone, which are responsible for the pharmacological activity (Ríos et al., 2002). All the active compounds mentioned here show similarities to 17β-estradiol (Figure 4), the endogenous ligand of the estrogenic receptor (ER) in mammals.

Endogenous estrogens might be competitively displaced at the ER by phytoestrogens, which would thus act as an anti-estrogen (Viereck et al., 2005). The isoflavones, for example, have less affinity than estradiol for the ER and in general, their affinity for binding to ERβ is 30 times higher than their affinity for ERα (Barkhem et al., 1998). Since ERβ are concentrated in the central nervous system, bones and the vascular wall, phytoestrogens will have a greater effect in these organs, while in the breast and endometrial tissues, where ERα are predominant, they will have less of an effect. In any case, as a consequence of their effects, phytoestrogens have been shown to improve the symptoms in postmenopausal women, especially the lipidic balance, thereby lowering cardiovascular risk. These effects are increased by the antioxidant properties of isoflavones (Anderson et al., 1995). However, the effects on bone and cancer have not yet been demonstrated in clinical trials; therefore, new and well-designed studies need to be carried out.

Black cohosh (*Cimicifuga racemosa* (L.) Nutt., syn. *Actaea racemosa* L.) has been used to treat the physiological signs of menopause for over 100 years (Mahady, 2005). Clinical trials on the effects of black cohosh extracts on menopausal symptoms have yielded positive results, with recent studies showing not only excellent efficacy against classic menopausal complaints, but also osteoprotective properties. Moreover, the extracts were deemed safe even when the dosage was increased threefold. Furthermore, several studies suggest that *Cimicifuga racemosa* extracts might help control the physiological and psychological problems typically found during menopause, such as hot flashes, profuse sweating, insomnia, and anxiety (Mahady, 2005; Viereck et al., 2005). However, the methodology used to

date lacks rigour and further clinical studies are needed. Moreover, some transient adverse events have been observed and recent experimental data suggest that cimicifuga extracts are, in fact, not estrogenic (Mahady, 2005).

Medicinal plants and benign prostatic hyperplasia

There are various pathologies and dysfunctions that specifically affect men, such as benign prostatic hyperplasia, prostatitis, prostate cancer and erectile dysfunction, together with other problems that affect men and women alike, such as urolithiasis or urinary tract infections. In this section we will review the specific developments in the treatment of benign prostatic hyperplasia with medicinal plant extracts. Benign prostatic hyperplasia is a non-malignant enlargement of the prostate that can lead to obstructive and irritative lower urinary tract symptoms. The pharmacological use of phytotherapy for the treatment of lower urinary tract symptoms associated with benign prostatic hyperplasia has been growing steadily in the past few years.

There is a great deal of evidence of the positive effects on prostate pathologies of diets with low levels of saturated fats but rich in fibre, antioxidants, phytoestrogens and phytosterols (Dreikorn, 2005). These data, in part, support the use of medicinal plants against prostate pathologies, but good results have been observed specifically with saw palmetto (*Serenoa repens* Barthram, syn. *Sabal serrulata* Nutral ex Schultes) and African prune tree (*Pygeum africanum* Hook, syn. *Prunus africana* Hook f. Kalkm.). Still other plants are commonly used to treat prostate problems in folk medicine or phytotherapy, including African potato (*Hypoxis rooperi* T. Moore, roots), stinging nettle (*Urtica dioica*, roots), rye (*Secale cereale* L., pollen), and pumpkin (*Cucurbita pepo* L., seeds). The active fractions generally contain phytosterols, triterpenoids, lipids and fatty acids, but other compounds have been identified, such as phytoestrogens, lectins, and polysaccharides (Dreikorn, 2005).

The *n*-hexane lipidosterolic extract of saw palmetto has been included in a large number of clinical trials and its efficacy in the treatment of benign prostatic hyperplasia has been demonstrated in placebo-controlled, comparative clinical studies. However, its mechanism of action has not yet been clearly described, although an anti-androgenic action, an anti-inflammatory effect and an antiproliferative influence through the inhibition of growth factors are probably all implicated (Buck, 2004). Specific studies have demonstrated that the lipophilic extracts from saw palmetto produce an inhibition of 5α-reductase in a dose-dependent and non-competitive manner. Moreover, the extract inhibits the receptor binding of androgens, α_1-receptor binding and eicosanoid synthesis (ESCOP, 2003).

Clinical trials have demonstrated that a standardized preparation of African prune tree may be a useful treatment option for men with lower urinary tract symptoms consistent with benign prostatic hyperplasia. However, the studies were small in size and short in duration; they also used varied doses and preparations and rarely reported outcomes using standardized, validated measures of efficacy. Additional placebo-controlled trials are therefore necessary to test the efficacy of African prune tree extract on lower urinary tract symptoms related to benign prostatic hyperplasia (Wilt et al., 1998).

Medicinal plants and the central nervous system

Many medicinal plants have been shown to be effective against various afflictions related to the central nervous system. Tea, coffee, cola and guarana are stimulants due to their caffeine content, while the stimulants ephedra and coca contain ephedrine and cocaine, respectively. Opium, of course, is an analgesic and a depressant due to its morphine content. However, many species with specific activity have no one defined active principle. For example, there are several studies on valerian or passion flower in which some concrete constituents have been isolated, but their specific activity has not been demonstrated.

Recently, several acetylcholinesterase inhibitors were introduced in the pharmacotherapeutic treatment of Alzheimer's disease. Of the natural products used to treat this malady, galantamine has shown good potential. This substance, which is found in different Amarillidaceae plants, has been demonstrated to have significant benefits on cognitive and global measures relevant in dementia (Lleó et al., 2006). These results have opened the field to potential new medicinal plants with acetylcholinesterase inhibitors.

Valerian (*Valeriana officinalis* L.) and St. John's wort (*Hypericum perforatum*) are the two medicinal plants most widely used against troubles and diseases affecting the nervous system. Valerian root extract is used for its hypnotic and sedative properties to provide relief of temporary mild nervous tension and/or difficulty in falling asleep (ESCOP, 2003). Its effects have been widely demonstrated in different experimental *in vivo* studies and corroborated in various clinical trials. The extract was found to reduce the pre-sleep phase and improve sleep duration. Moreover, latency time was reduced and sleep phases increased with no alteration to REM sleep (Houghton, 1999).

Hydroethanolic extracts from St. John's wort are indicated to treat episodes of mild depressive disorders or mild to moderate depressive episodes. Other preparations are recommended to treat mild depression and support emotional balance (ESCOP, 2003). The clinical efficacy of

various standardized extracts in the treatment of depression has been demonstrated in 38 controlled clinical trials and two recent meta-analyses. One conclusion of these studies was that the safety of the extracts was more favourable than that of synthetic antidepressants. However, pharmacokinetic interactions may occasionally occur as a result of changes in the activity of drug-metabolizing and drug-transporting proteins. Consequently, St. John's wort preparations should not be taken concurrently with other antidepressants, coumarin-type anticoagulants, the immunosuppressants cyclosporine and tacrolimus, protease and reverse transcriptase inhibitors or antineoplastic agents (Schulz, 2006).

Another medicinal plant widely used for treating Alzheimer's disease is ginkgo (*Ginkgo biloba*), several standardized extracts of which are used in official medicine. Although the efficacy of ginkgo is low, it has a high degree of safety (Lleó et al., 2006). It is mostly used to reduce or prevent memory deterioration due to ageing and milder forms of dementia, including Alzheimer's disease (Heinrich et al., 2004).

Different trials and systematic reviews have demonstrated the efficacy of ginkgo for the symptomatic treatment of dementia and suggested that it is effective in delaying the clinical deterioration of patients or in bringing about symptomatic improvements (Ernst, 2005). The same author reached a similar conclusion concerning the use of ginkgo extracts in the treatment of tinnitus, concluding that the evidence was favourable although methodological limitations make clear conclusions difficult.

Free radicals or oxidative injury is one of the causes of human neurological diseases; therefore, therapies that make use of free radical scavengers have been postulated to prevent, delay or ameliorate these pathologies. However, clinical trials with various antioxidants have not been conclusive and more specific studies need to be carried out (Delantry and Dichter, 2000).

Medicinal plants and modification of the immune response

The use of medicinal plants for enhancing the immune response has been reported by different authors (Labadie et al., 1989). However, the degree of effectiveness of many such plants has not been demonstrated. The principal species that have been studied in this field to date are *Echinacea purpurea* (purple coneflower), *E. angustifolia* DC (narrow-leaf coneflower), *E. pallida* Nutt. (pale purple coneflower), and *Astragalus membranaceus* (Fisch.) Bunge, all of which have been used for centuries for treating the common cold, coughs, bronchitis and inflammation of the mouth and pharynx.

Echinacea is the most common immunostimulant plant used in phytotherapy, and its properties have been affirmed by various *in vivo* and

in vitro experiments. Such studies have demonstrated a wide range of effects, including increases in phagocytosis, chemotaxis and oxidative burst of both neutrophils and macrophages. Moreover, it is toxic against various tumor cells and cells infected with the parasite *Leishmania enriettii* or the yeast *Candida albicans*. However, since the results vary when different preparations or species are used, it is necessary to run these experiments with a defined species and standardized preparations. The clinical trials to date have not been conclusive, probably due to the aforementioned problems in methodology, but the properties of Echinacea seem to be therapeutic, not prophylactic (Percival, 2000).

Astragalus membranaceus and other closely related species have also been shown to have immunostimulant properties and they are widely used in traditional Chinese medicine to improve the immunological response against different infections and cancer. Their activity has been related to the polysaccharide fraction, and two active compounds have been identified (astragalan I and II); however, other compounds such as astragalosides may also be implicated (Ríos and Waterman, 1997). Several clinical studies carried out in China have demonstrated the plant's immunostimulant properties in colds and upper respiratory infections (Heinrich et al., 2004).

Medicinal plants and human exercise performance

Adaptogens or inducers of states of non-specifically increased resistance are the agents that exert effects on both sick and healthy individuals by correcting any dysfunctions without producing unwanted side effects (Davydov and Krikorian, 2000). Various medicinal plants are used to this end, including *Cordyceps sinensis* (Berkeley) Saccardo, *Eleutherococcus senticosus*, *Lepidium meyenii* Walp., *Panax ginseng*, *P. notoginseng* F.H. Chen ex C.Y. Wu & K.M. Feng, *P. qinquefolius* L., *Pfaffia paniculata* (Mart) O. Kuntze, *Rhodolia crenulata* (Hook. & Thomson) H. Ohba, *Schizandra chinensis*, *Smilax officinalis* Kunth. and *Withania somnifera* (L.) Dunal (Bucci, 2000; Davydov and Krikorian, 2000). Of these, *Eleutherococcus senticosus* and *Panax ginseng* are those that have been studied the most and whose effects have been documented the best.

Some medicinal plants, especially ginseng, have been reported to have aphrodisiac properties. Indeed, in a clinical study of patients with erectile dysfunction, changes in early detumescence and erectile parameters such as penile rigidity and girth, libido and patient satisfaction were significantly higher in ginseng-treated patients than in both the reference group and those given a placebo. This effect is related to the release of NO from both endothelial cells and perivascular nitrergic nerves, with the compound responsible for this release most likely being ginsenoside Rg3

(Gillis, 1997; Achike and Kwan, 2003).

A second group of herbs and plant extracts are used as ergogenic aids to enhance long-term endurance, increase muscular strength and boost athletic performance (Bucci, 2000). This group includes adaptogenic plants as well as species with caffeine (*Coffea arabica* L., *Camellia sinensis*, *Paullinia cupana* Mart. and *Ilex paraguariensis* Saint Hilaire) and plants with ephedrine and related alkaloids (*Ephedra sinica* Stapf, *Citrus aurantium* L. and *Sida cordifolia* L.) (Bucci, 2000). The combination of plant extracts with caffeine and ephedrine or *p*-synephrine produces improvements in physical performance that are higher than those observed for caffeine or ephedrine alone. This combination may also reproduce short-term thermogenic and metabolic effects that lead to the loss of body fat (Bucci, 2000).

Perspectives

Could biotechnology be applied to medicinal plants and their active principles? This seems to be a valid possibility to avoid the typical problems associated with obtaining such substances. As mentioned above, medicinal plants are a promising source for the development of drugs, and many types of active ingredients from plant sources have been studied in order to clarify the relationship between their chemical structure and their activity; however, the limited quantity of active compounds in a given plant or plant organ, along with the low growth rate of many plants leads to problems in the development of this type of research. In such cases, plant cell cultures may be a useful technology in solving these problems as they would secure a stable supply of the active compounds without damage to natural plant resources. This was the method followed for the efficient production of the anticancer drug paclitaxel. *Taxus* cell suspension cultures were used because these cell lines were found to produce paclitaxel and related taxanes upon being subjected to specific external stimuli. By applying the data from both the two-stage culture and the high-density culture, a large-scale culture process was developed which afforded a stable paclitaxel production in the range of 140-295 mg/l (Tabata, 2006).

Vinblastine and the related alkaloid vincristine from *Catharanthus roseus* are some of the best-studied principles from medicinal plants. Due to their pharmaceutical importance and their low content in the plant, a model system was developed for biotechnological studies on the secondary plant metabolism, in which the alkaloid biosynthetic pathway can be manipulated genetically by 'metabolic engineering' to achieve higher production levels of *Catharanthus* alkaloids (Van der Heijden et al., 2004).

The study of natural products from fungi could be of even greater

interest for the application of biotechnology. Recently, *Agaricus blazei* Murill and *Ganoderma lucidum* (Curtis Fr.) P. Karst. have been used for the treatment of cancer and several active substances isolated from them have served as antitumor and antimetastatic agents (Kimura, 2005). These active substances obtained from basidiomycete could be produced by means of biotechnology.

While the use of new medicinal plants is welcome, the reintroduction of known plants on which research was halted for various reasons is of great interest for therapies based on natural products and plant extracts derived from natural sources. Such is the case of cannabis (*Cannabis sativa* var *indica*) and cannabinoids. The selection of adequate non-psychotropic extracts or structural modifications of the active principles could be useful for the introduction of new active substances and extracts. This is the case of nabilone as an anti-emetic and of benzopyranoperidine, levonantradol and ajulemic acid as analgesics. The preparation of adequate extracts with reduced side effects could be effective in reducing nausea and vomiting, in stimulating appetite and in reducing different types of pain in the treatment of multiple sclerosis, spinal cord injuries, Gilles de la Tourette's syndrome, epilepsy, glaucoma, Parkinson's disease and dystonia (Ben Amar, 2006). Several clinical trials on patients with these ailments have produced good results, especially in the treatment of refractory emesis. However, further clinical trials—well-designed, carefully executed and powered for efficacy—are essential for defining the specific role of cannabionoids, as well as for defining the best administration route in order to maximize the beneficial effects of each preparation and minimize undesirable reactions (Ben Amar, 2006).

Finally, the effective use of medicinal plants and phytomedicines for treating certain pathologies demands the use of a defined protocol for the preparation of extracts, well-established standardization methods, pharmacological studies of the elaborated phytomedicine and not merely of the active plant, specific clinical trials and good, systematic reviews on the utilization of these substances (Tyler, 1999). One of the main stumbling blocks to this type of research is the difficulty in and high cost of developing clinical trials to test the effects of medicinal plants and plant extracts. In most cases, the larger entities that market drugs are willing to make a substantial investment in clinical studies, but only for their own products. Meanwhile, smaller companies do not have the financial resources to sponsor this kind of research. Another problem in the development of clinical trials is that associated with standardization and the determination of phytoequivalence. The fact is that many medicinal plants such as chamomile or echinaceae have no one defined active principle. In this case, a group of principles, the essential oils (chamomile and valerian) or flavonoids (ginkgo), for example, is taken to be the

reference for the sake of the quality control. In the case of St. John's wort extracts, the European Pharmacopoeia refers to hypericin as a marker although the active principle has not been defined. In addition, bioavailability studies are recommended in order to gain insight into the behaviour of an extract after its administration. Solving these problems, together with the adequate use of appropriate excipients and diluents, could improve the therapeutic properties of many phytomedicines (Tyler, 1999).

Conclusions

Since their reintroduction as a valid therapeutic system in Western medicine, the activity and safety of medicinal plants have been widely studied. More recently, pharmacological studies have demonstrated the specific properties of a great number of these plants. However, the phytotherapy of the future should be based on the efficacy of medicinal plants together with their extracts and products. For this purpose, the inclusion of greater numbers of plants in possible clinical trials is desirable. But such trials need be of a sufficient size and duration to detect important differences in clinically relevant endpoints. They also need to use standardized symptom scale scores. Thus, selection of randomized controlled trials involving a higher number of patients, double-blinded experiments, and the use of standardized extracts with standard drugs as a positive control should all be established practice for the selection of phytomedicines for their therapeutic use.

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11 Cannabimimetics: The Pharmacological Potential of Cannabinoid Receptor Ligands

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Introduction

Indian hemp (*Cannabis sativa* L. var. *indica*) from the Cannabaceae family has been used and cultivated since ancient times for therapeutic or ritual purposes. The Indian hemp plant (Figure 1) was originally considered as a distinct species but came to be regarded as a variety of *C. sativa*, the common European hemp, which exhibits a variety of ecotypes giving rise to plants with differing chemical compositions. One of the earliest historical accounts of the medicinal use of *Cannabis* is found in the Chinese pharmacopoeia from 200 A.D. (Lambert and Fowler, 2005). Throughout human history *Cannabis* herba (marijuana) has been used because of its psychomodulatory effects, but also to treat pain, nausea, fever, infections, stimulation of appetite, and for gynaecological disorders. The Chinese emperor Huang Ti apparently adviced taking *Cannabis* for the relief of muscle cramps, and rheumatic and menstrual pain as early as 2600 B.C. (Di Marzo et al., 2004). The Roman physician Claudius Galenus [129-199 A.D.] was the first to mention the narcotic effect of *Cannabis* (Tschirch, 1910). Much has been written about the psychotropic effects of *Cannabis* flower resin (hashish), e.g. about its use as a psychomimetic drug by the Assassins (*hashishin*, meaning user of *Cannabis* resin). Led by Hasan Al Sabah, the 'Old Man of the Mountain', the Assassins were a secret society active during the Middle Ages and based at the mountain fortress of Alamut, located in modern-day Iran (Bromberg and Rodgers, 1946). Members of this society extensively consumed *Cannabis* to relax and pacify after warfare, and apparently also to subordinate the novices (Tschirch, 1910). The

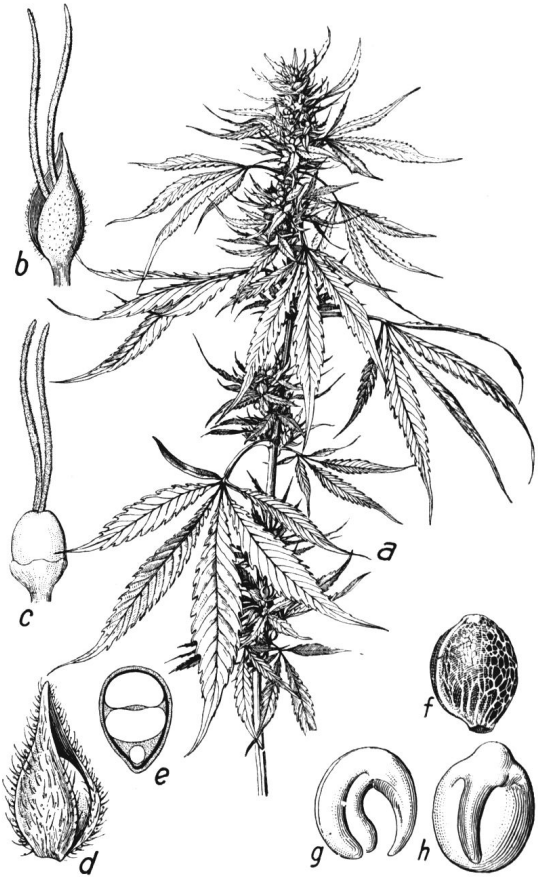


Fig. 1. *Cannabis sativa* L. female plant. *a*—habitus, *b-c*—female flowers, *d*—pseudofruit, *e*—cut through seed, *f*—seed, *g-h*—embryo (adapted from Karsten & Weber, *Lehrbuch der Pharmakognosie*, 1926).

narcotic effect of marijuana derived from *Cannabis sativa* var. *indica* was known in India and the Islamic countries of the East, probably because this was also the origin of the *Cannabis* varieties rich in narcotic principles. In Europe, *Cannabis* has been primarily used for fibre production but also also as medicine. In his 'The Complete Herbal' the English physician Nicholas Culpeper [1616-1654] summarized all the conditions for which *Cannabis* was supposed to be medically useful. The New English Dispensatory of 1764 recommended applying hemp roots to the skin for inflammation, a remedy that was already popular in Eastern Europe. The Edinburgh New Dispensary of 1794 included a long description of the

effects of hemp and stated that the oil was useful in the treatment of coughs, venereal disease, and urinary incontinence. But *Cannabis* did not become popular as a medicine until the middle of the 19th century. During its early heyday, from 1840 to approximately 1900, more than 100 papers were published in the Western medical literature, recommending *Cannabis* for various illnesses and discomforts (Mikuriya, 1973). Little is known of the recreational use of *Cannabis* in Europe prior to its introduction as a narcotic by Napoleon's soldiers returning from Egypt where, at that time, marijuana was smoked mainly by the underprivileged (Kimmens, 1977). In the 19th century *Cannabis* was increasingly consumed by artists and intellectuals as a mind opening entheogen (Baudelaire, 1858). Famous for recreational *Cannabis* experimentation was the 'club of haschischins' founded by Baudlaire, Gautier, and Rimbaud on one of the Seine isles in France (Gautier, 1843). Since the 1960s marijuana has become the most widespread illicit drug of abuse in the world.

Despite major technological and conceptual advances in biochemical pharmacology during the second half of the 20th century, the progress of the knowledge on *Cannabis* pharmacology was rather slow. Moreover, the initial focus in *Cannabis* research was almost exclusively on the psychotropic effects, and the concept of the peripheral cannabinoid receptors was unknown. The psychoactive principle of *Cannabis* was described as an oil mixture and was referred to as cannabinol (Tschirch, 1910). In the early 1940s cannabidiol and cannabinol, the first pure cannabinoids, were isolated and investigated chemically. Since these compounds were evidently not the psychoactive principles of marijuana (female flowers of *C. sativa*), despite of cannabinol being able to act on cannabinoid receptors as found later, they were largely ignored. In 1964, Gaoni and Mechoulam finally identified Δ^9-tetrahydrocannabinol (Δ^9-THC) as the major psychoactive principle in *Cannabis*, ending a search for almost 60 years. The fascinating history of the chemical research on plant cannabinoids has been reviewed (Mechoulam and Hanus, 2000; Russo, 2007) and is not a subject of this chapter. Until the 1980s, the term cannabinoids represented, by definition, the group of typical C21 compounds present in *Cannabis sativa* L., their carboxylic acids, and transformation products also referred to as phytocannabinoids (Lambert et al., 2005). The most important phytocannabinoids are Δ^9-THC, Δ^8-THC, cannabidiol, and cannabinol (Figure 2). Despite the knowledge about Δ^9-THC as the major bioactive principle its cellular target was not identified for more than 25 years. Early cannabinoid research mainly focused on behavioural effects in animals, and structure-activity relationship studies employed the dog ataxia test, experiments with rhesus monkeys, or the 'cannabinoid tetrad test battery' which is generally performed in mice (Martin, 1986). The lack of a biological target protein for Δ^9-THC during

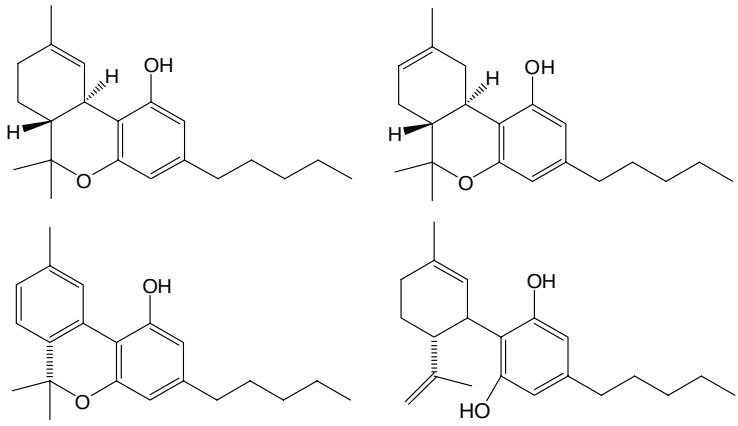


Fig. 2. Major chemical constitutents (classical cannabinoids) of *Cannabis sativa* L. var. *indica.*

that time of research led to much controversy between scientists as some speculated that non-specific toxic effects on cellular membranes could account for the psychomimetic action of the lipophilic compound Δ^9-THC.

Since the discovery that Δ^9-THC and related cannabinoids act on specific physiological receptors in the human body, and the subsequent elucidation of the mammalian endogenous cannabinoid system, the field of cannabinoid research has continually expanded. The first report of a specific cannabinoid receptor, which binds Δ^9-THC was published in 1988 (Devane et al., 1988). This initial study was performed with the tritiated Pfizer compound CP-55,940 (**2**), a ring-opened and less lipophilic analogue of Δ^9-THC which competitively binds to the same receptors as Δ^9-THC (Howlett et al., 1990). Tissue binding studies with [^{3}H]CP-55,940 suggested that the cannabinoid receptor density was greatest in the limbic and cortical areas, the basal ganglia and cerebellum, and that it gradually decreased into the brainstem and spinal cord (Herkenham et al., 1990, 1991; Mailleux and Vanderhaeghen, 1992). Subsequent to this, the first cannabinoid receptor, the cannabinoid type-1 (CB_1), was cloned from a rat brain cDNA library using a probe derived from the sequence of bovine substance-K receptor (Matsuda et al., 1990). After the cloning of the CB_1 receptor subsequent DNA sequence homology studies led to the discovery of a second cannabinoid receptor (the so-called type-2 or CB_2 receptor) from the myelomonocytic leukemia cell line HL60 (Munro et al., 1993).

Cannabinoid receptors belong to the class A rhodopsin-like G-protein-coupled receptor (GPCR) family. The CB_1 receptor, which has been cloned from human, cat, rat, and mouse is predominantly but not exclusively found in the nervous system, while the CB_2 receptor, which has been cloned

from human, mouse, and rat is mainly expressed on immune cells and in the spleen (Cabral and Marciano-Cabral, 2005; Mackie, 2005). Even though the classification of CB_1 as central and CB_2 as peripheral cannabinoid receptors is generally correct it is important to note that the CB_1 receptor can be strongly expressed in certain peripheral cells like immune cells and that the CB_2 receptor is potentially also present in neurons of the brain (Gong et al., 2006). The cannabinoid receptors show approximately 48% overall identity with 68% identity in the transmembrane regions (Howlett et al., 2002), and are coupled to the G_i/G_o family of G-proteins (Howlett, 2005). Signal transduction pathways regulated by cannabinoid receptors include the inhibition of adenylate cyclase and stimulation of phospholipase C (*vide infra*).

The presence of cannabinoid receptors in mammalian cells was indicative of the existence of an endogenous cannabinoid system. One of the first clues to the existence of cannabinoid receptors was provided by studies showing that incubation of neuroblastoma cells with cannabinoids included a decrease of cyclic adenosine monophosphate (cAMP) in the cells, suggesting that the putative receptors were negatively coupled to adenylate cyclase. *N*-arachidonoyl ethanolamine, the first endogenous ligand described for the cannabinoid receptors, was discovered in the early 1990s (Devane et al., 1992; Di Marzo et al., 1994; Thomas et al., 1996). This arachidonic acid derivative was called 'anandamide', referring to the Sanskrit term 'ananda', meaning bliss. Anandamide, which is synthesized on demand activates both CB_1 ($K_i < 50$ nM) and CB_2 ($K_i < 250$ nM) receptors and is rapidly eliminated through a two-step process consisting of a putative carrier-mediated transport followed by metabolization by the membrane-bound serine hydrolase, referred to as fatty acid amide hydrolase (FAAH) (Ligresti et al., 2005). It soon became apparent that anadamide was only one member of a whole family of endocannabinoids and that other endogenous cannabinoids, including 2-arachidonoyl glycerol (2-AG) together with CB_1 and CB_2 receptors constituted a major neurochemical and possibly also immunomodulatory system in mammalian physiology (Mechoulam et al., 1998; Klein et al., 2003; Di Marzo et al., 2007). The search for endogenous ligands for the cannabinoid receptors has led to the discovery of several polyunsaturated compounds derived from fatty acids, mostly arachidonic acid (Kogan and Mechoulam, 2006). However, if endocannabinoids are defined as being part of distinct synthetic and metabolic pathways, and capable to bind to the cannabinoid receptors selectively at physiological concentrations, only anandamide and 2-AG are true endogenous cannabinoids. The physiological role of anandamide is still unclear and its action appears to be associated to areas as distinct as schizophrenia and reproduction. Moreover, the question remains whether the numerous effects reported for anandamide merely reflect its

action on CB_1 and CB_2 receptors or whether other yet unknown targets may also play a role.

The discovery that anandamide binds to cannabinoid receptors and acts as an endogenous mimic of Δ^9-THC led to a pronounced interest in other fatty acid amides as cannabinoid receptor ligands (Appendino et al., 2006a, 2006b; Di Marzo, 2007). As reviewed recently, several groups have explored the synthesis and biological evaluation of anandamide analogues, obtained by modifications of the fatty acid chain and of the ethanolamine head group (Mahadevan and Razdan, 2005). Based on the structure of anandamide efforts have been undertaken to design ligands with improved binding selectivity for individual cannabinoid receptors and with a better pharmacokinetic profile (Kogan and Mechoulam, 2006). Our recent finding that the medicinal plant purple coneflower (*Echinacea purpurea* and *E. angustifolia*), which is indicated for the treatment of upper respiratory infections and the common cold, produces *N*-alkyl amides which are high-affinity functional CB_2 receptor-specific ligands (K_i ~ 50 nM) has led us to search for other CB_2 binding compounds within this natural product family. Since fatty acid amides are relatively abundant in plants, these hitherto neglected natural products may be a feasible source for new cannabinoid lead structures and model compounds to study CB_2 receptor signal transduction. In our screening for CB_2 binding compounds we have recently identified distinct CB_2 binding *N*-alkyl amides from different plant taxa as well as the ubiquitous sesquiterpene beta-caryophyllene that also exerts cannabimimetic effects both *in vitro* and *in vivo* (Gertsch et al., 2006; Gertsch et al., 2008).

In this chapter the pharmacological relevance of cannabinoid receptor ligands is reviewed, with a special focus on recent developments in the field of CB_2 cannabinoid receptor drug discovery.

CB_1 receptor pharmacology

The pharmacology of cannabinoid receptors, especially CB_1 receptors, has been reviewed extensively (Klein et al., 2003; Di Marzo et al., 2004; Howlett, 2005; Lambert and Fowler, 2005; Mackie, 2006). The CB_1 cannabinoid receptor is highly conserved (for a review see Lutz, 2002), and it has been cloned from several species, including zebra fish and amphibian newt. Interestingly, it seems to be absent in insects and sponges, and cannabinoid receptors are thus believed to have evolved in the last common ancestors of bilaterians, with secondary loss occuring in insects and other clades (McPartland et al., 2005). In mammals CB_1 receptors are not only expressed in the brain but also testis, ileum, urinary bladder, the vas deferens, and on immune cells of the monocytic lineage (unpublished observation). Alternatively spliced forms of the CB_1 cannabinoid receptor have been

found (Ryberg et al., 2005), though their role is still unknown. In the CNS, CB_1 receptors are predominantely expressed on axons and nerve terminals, and functional evidence also supports their expression on somata (Howlett et al., 2002). In man, the CB_1 receptor is responsible for the 'high' from marijuana smoking produced by centrally available Δ^9-THC. Cannabinoid receptors are G-protein coupled receptors (GPCR), which are linked to adenylate cyclase (ADC) and a variety of other second messengers and signalling components. Ligation of CB_1 has been shown to not only suppress ADC but also to activate it (Rhee et al., 1998). CB_1 receptors also inhibit presynaptic N- and P/Q-type calcium channels and activate inwardly rectifiying potassium channels (Mackie et al., 1995; Twitchell et al., 1997). Smoked doses of 20 mg of Δ^9-THC will produce increases of 20 to 50 beats per minute in heart rate, impairment of short term memory and concentration, possibly mood changes such as euphoria, altered perception of time, hunger, and other effects. At higher doses, marijuana interferes with motor coordination and complex task functioning. Pharmacological effects from inhaled Δ^9-THC and Δ^8-THC begin almost immediately. Δ^9-THC plasma levels peak 10 to 20 minutes post inhalation and begin to decline. Effects last for two to three hours. Studies indicate that marijuana and alcohol potentate one another more than either drug alone. Thus, smaller amounts of ethanol and marijuana may increase toxic effects. Peak levels of marijuana metabolites in the urine occur about five hours post dose and thus are not useful in predicting the degree of intoxication. Knockout animals have been obtained for the CB_1 receptor by several groups (Zimmer et al., 1999; Ledent et al., 1999). The CB_1 receptor predominantely mediates the antimimetic action of *Cannabis* (Mechoulam, 1986) and agonists, such as Δ^9-THC (Dronabinol, Marinol™) are used to reduce vomiting induced by anticancer chemotherapy (Plasse, 1991). Of all potential drugs affecting the endocannabinoid system, CB_1 receptor antagonists have probably received the most attention because of the recently marketed and subsequently withdrawn drug Acomplia™ (rimonabant) (**1**), also known as SR141716, indicated for adipositas (obesity). The drug was developed based on the observation that marijuana consumption stimulates the appetite. Thus, the rationale for using a CB_1 antagonist is conceptually simple as blocking the receptor might decrease the appetite. In fact, CB_1 antagonist decrease weight, but not for quite these reasons as the weight loss involves metabolic effects in hepatocytes and adipocytes (for a review see Mackie, 2006). Clinical studies with CB_1 antagonists are generally perceived as encouraging and rimonabant (**1**) at a daily dose of 20 mg together with a hypocaloric diet leads to sustained weight loss of an average of 7 kg versus a weight loss of 2 kg in the placebo treated group (Van Gaal et al., 2005). Although this may appear as a modest result, rimonabant also led to significant improvements in lipid profiles and CB_1

antagonists have been shown to block the increase in fatty acid synthase expression induced by a high-fat diet (Osei-Hyiaman et al., 2005). Previuos to this finding, a molecular connection between the endocannabinoid system and fatty acid synthesis had already been established (Osei-Hyiaman et al., 2003). In adipocytes, CB_1 activation has been associated to lipoprotein lipase activity (Cota et al., 2003).

For some time it was assumed that the endocannabinoid system may regulate addictive behaviour (for a review see Mackie, 2006). From a large number of animal experiments, there appears to be a reciprocal relationship between the motivational or rewarding aspects of endogenous opioids and the endocannabinoids (Corchero et al., 2004). In fact, the rewarding properties of opioids are absent in CB_1 knockout mice (Cossu et al., 2001). During the trials with **1** the link between the CB_1 receptor and addictive behaviour could be confirmed (Gallate et al., 2004). Whether CB_1 antagonists should be considered in the treatment of craving disorders depends on the overall behavioural effects of CB_1 antagonists. The apparent involvement of the CB_1 receptors in addiction, such as nicotine and alcohol, has been the subject of many studies. CB_1 antagonists block nicotine-induced conditioned place preference and dopamine release in the nucleus acumbens (Valjent et al., 2002). Moreover, CB_1 receptor activation has been reported to enhance alcohol consumption while blocking or deletion of CB_1 decreases alcohol consumption (Gallate et al., 2004). Thus, other potential applications of CB_1 antagonists are treatment of nicotine and alcohol addiction. The recent clinical approval and worldwide marketing of Acomplia™ will now enable clinicians to determine whether CB_1 antagonists confirm the promising therapeutic value for the treatment of craving disorders. Due to the possible role of CB_1 receptors in the regulation of anxiety, memory extinction, and schizophrenia there is a theoretical concern that chronic CB_1 blockage might be accompanied by significant mood disorders. Although clinical experience with Acomplia™ is merely based on two years human trial only modest gastrointestinal problems, dizziness, and arthralgias have been reported (Van Gaal et al., 2005). Since the CB_1 receptor is likely to play multiple roles, such as control of inflammation in the CNS and regulation of bone development (*vide infra*), its blockage may well have long-term side effects.

In a recent article Teixeira-Clerc et al. (2006) reported that CB_1 receptors are strongly induced in fibrogenic liver cells, such as stellate cells of the cirrhotic human liver and in stellate cells of mice treated with carbon tetrachloride, a known fibrinogenic stimulus. The antifibrinogenic effect of CB_1 blockage could be attributed to decreased proliferation and increased apoptosis of activated stallate cells, probably related to a reduction in the hepatic levels of the dominant fibrinogenic cytokine transforming growth factor-β-1 (TGF-β1). These findings raise the possibility of using CB_1

antagonists to treat liver cirrhosis (Kunos et al., 2006).

The effects of Δ^9-THC which are mediated via CB_1 activation have been extensively studied and characterized. Nonetheless, data about the detrimental effects of Δ^9-THC are contradictory. The abuse of marijuana and its purified products has been assosiated with modulation of cognition and profound psychological changes. Although the psychotropic effects of Δ^9-THC are generally accepted there is a controversy about its addictiveness. Promising results in the use of Δ^9-THC in cancer patients, particularly in the relief of nausea and vomiting caused by chemotherapy have led to the development of Δ^9-THC based medications, such as dronabinol (synthetic Δ^9-THC), which is used for the treatment of nausea and vomiting. In the US, together with its use to stimulate the appetite of AIDS patients, Δ^9-THC is also indicated for the treatment of the symptoms of multiple sclerosis. Related to the medicinal use of *Cannabis* is the recent introduction of the standardized *Cannabis sativa* extract Sativex™, which contains Δ^9-THC, cannabinol, the non-psychoactive cannabidiol (Smith, 2004). Moreover, Sativex™ also contains full terpenoid content, such as mono and sesquiterpenes. Since Δ^9-THC and cannabinol not only activates CB_1 but also CB_2 receptors (*vide infra*) it is not known whether the positive pharmacological effects of these compounds are mediated by CB_1, CB_2, or a combination of both. Since CB_2 receptor agonists have been shown to produce many of the beneficial effects reported for Δ^9-THC, but lack the psychotropic side effects, cannabimimetics with CB_2 selectivity over CB_1 are currently being developed for therapeutic use.

CB_2 receptor pharmacology

Overall, the exact physiological role of CB_2 receptors remains to be fully defined. However, several intriguing preclinical studies suggest that CB_2 agonists may be clinically useful for distinct disease areas (Figure 3). In fact, the potential use of CB_2 receptor agonists has been strongly emphasized in different recent reviews (Lambert and Fowler, 2005; Mackie, 2006).

In addition to its role in the cellular immune system (e.g. resolution of inflammation) (Klein et al., 2003), the CB_2 receptor has become an attractive target for drug discovery in areas as distinct as chronic pain, atherosclerosis, and osteoporosis (*vide infra*). Recent studies also highlight the potential of CB_2 receptor specific ligands in the treatment of cancer, including tumors of the immune system (Alberich Jorda et al., 2004; Sarfaraz et al., 2005; www.pharmoscorp.com) and brain tumors like gliomas (Blázquez et al., 2008). However, despite a wealth of preclinical data only the specific synthetic CB_2 agonist cannabinor (PRS-211,375) (**3**) from Pharmos Ltd. is in phase II clinical trial as an analgesic for pain and

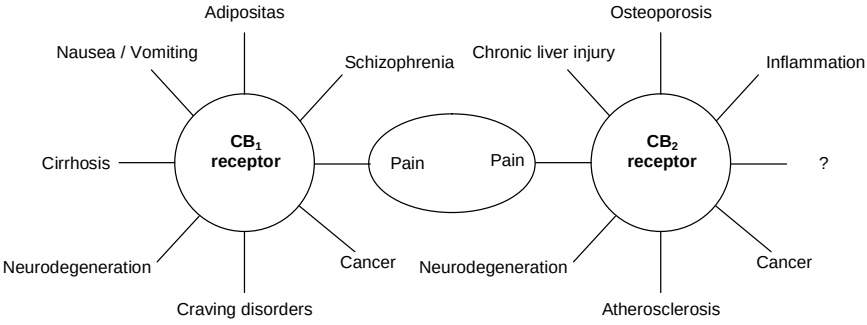


Fig. 3. Cannabinoid receptors have been associated with distinct pathophysiologies and have therefore been discussed as possible targets for a range of diseases.

inflammation (www.pharmoscorp.com). This compound has also been found to be efficacious in preclinical models of multiple sclerosis, rheumatoid arthritis, and inflammatory liver damage (www.pharmoscorp.com; Lavon et al., 2003). In light of such developments it is not surprising that several laboratories now focus on the elaboration of new CB_2 ligands. Therefore, not only CB_1-binding compounds, like the successful phase III Sanofi CB_1 antagonist Acomplia™ (*vide supra*) or the clinically used unselective CB_1/CB_2 agonists Marinol™ (dronabinol), and Cesamet™ (nabilone), but also compounds specifically directed at the CB_2 receptor are promising candidates for drug discovery. At present, potent antagonists for both receptors, as well as four major structural classes of agonists have been identified (*vide infra*). While an extensive amount of structure-activity relationship (SAR) information has been developed for CB_1 ligands (*vide supra*), the SAR for CB_2 ligands is only now emerging in the literature (Lambert and Fowler, 2005; Reggio, 2005).

A particularly attractive feature of selective CB_2 agonists as potential therapeutics is the fact that they are devoid of psychomimetic activity. It is well known that CB_1 receptor activation typically leads to analgesia in different models (Hohmann et al., 2004) and *Cannabis sativa* and its extracts have been used to treat painful conditions for centuries. At the same time it is now also clear that selective CB_2 agonists are likewise effective in chronic pain models such as models of neurophatic pain, peripheral inflammatory pain, and also in some pain sensitization models (Ibrahim et al., 2003; Hohmann et al., 2004; Ibrahim et al., 2006). The sites of CB_2 analgesic action remain unclear, although the skin is likely to be one site as it has recently been shown that CB_2 agonists enhance β-endorphin release from keratinocytes (Ibrahim et al., 2005). According to Mackie (2006), the studies on the analgesic effects of CB_2 agonists in neurophathic pain

models following nerve injury suggest that the analgesia is mediated via spinal microglia (Wotherspoon et al., 2005).

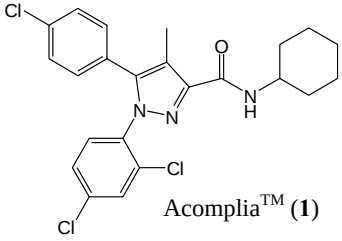
That CB_2 receptors are involved in bone growth has been impressively shown by the observation that CB_2 knockout mice show markedly decreased bone mass compared to wild type (Ofek et al., 2006). Moreover, the finding that in humans a silent single nucleotide polymorphism in CB_2 correlates strongly with osteoporosis in women (Karsak et al., 2005) seems to substantiate the role of this receptor in bone formation. The therapeutic possibilities of these findings are further evidenced by the observation that the CB_2 agonist HU308 (**4**) decreases bone loss following ovariectomy in mice (Ofek et al., 2006). The combination of these results and those of the efficacy of CB_2 agonists in chronic pain associated to inflammation is interesting because anti-inflammatory drugs are currently associated to detrimental effects on bone density (Mackie, 2006). However, the exact role of CB_2 receptors during osteoclastogenesis and for osteoblast development remains to be elucidated as the available data are somewhat contradictory because also stimulation of osteoclast activity via both CB_1 and CB_2 has been reported (Idris et al., 2005). Currently, it is not clear whether an agonist or an inverse agonist leads to inhibition of osteoclastogenesis. CB_2 ligands may have therapeutic utility in chronic inflammatory diseases. As was demonstrated recently, low doses of Δ^9-THC ($h$$CB_2$ K_i = 36.4 ± 10 nM) slows the extension of atherosclerotic lesions in ApoE-/- mice via a CB_2 receptor-mediated mechanism (Steffens et al., 2005). CB_2 mediated signaling may also be involved in neurodegeneration associated with plaque development in Alzheimer's disease (Ramirez et al., 2005). Another study suggests that CB_2 receptors may play a role in the inflammatory response accompanying retroviral encephalitis, as may e.g. occur after HIV infection (Benito et al., 2005). These findings clearly show that CB_2 receptor pharmacology has developed into an exciting new area of research and that CB_2 specific ligands may eventually provide new drugs to treat old diseases. However, many fundamental questions remain to be answered and the CB_2 signaling pathways in both immune and neuronal cells are far from being understood. Specific CB_2 agonists, inverse agonists, and antagonists will be required as tools for the elucidation of the molecular mechanisms and signals involved in CB_2 pharmacology. Any new class of CB_2 binding compound may therefore provide new insights into the working mechanism of this receptor.

Current CB_2 receptor ligands

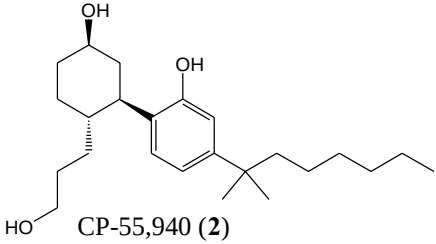
In general, cannabinoid ligands are classified as (a) classical cannabinoids related to Δ^9-THC, (b) the non-classical cannabinoids related to **2**, (c) the aminoalkylindoles related to WIN 55,212-2 (**5**), and (d) the eicosanoids

related to the endocannabinoids anandamide (**6**) and 2-AG (**7**) (Lambert and Fowler, 2005). Additional compounds, such as JTE907 (**8)** and BAY 38-7271 (**9**), which cannot be allocated into any of the standard classes have also been described (see Figures 4 and 5). Although many cannabinoid receptor ligands show little, or at best modest, selectivity for either receptor, a number of synthetic compounds are known which have significant selectivity for the CB_2 receptor (Figure 6). Prominent examples include HU308 (**4)**, AM1241 (**10**), the 1-methoxy Δ^8-THC derivatives, 1-methoxy Δ^9-THC-DMH (L759633) (**11**), 1-methoxy Δ^9(11)-THC-DMH (L759656) (**12**), plus a number of 1-deoxy Δ^9-THC analogues. In particular, 1-deoxy-3-(1',1'-dimethylbutyl) Δ^9-THC (JWH-133) (**13**) shows two hundred-fold selectivity for the CB_2 receptor over CB_1. In addtion, indol derivatives such as 1-propyl-2-methyl-3-(1-naphthoyl)indole (JWH-015) (**14**) or 1-(2,3-dichlorobenzoyl)-2-methyl-3-(2-[1-morpholino]ethyl)-5-methoxyindole (L768242) (**15**) have been reported as CB_2-specific cannabimimetics. Efforts have been made to develop structure-activity relationships (SAR) at CB_2 for cannabimimetic indoles, but with limited success. Derivatives bearing a cyclohexane ring ortho to the phenolic hydroxyl group of the phenol represent another major class of cannabinoid ligands. These compounds are related to the ring-opened pyran derivatives. Efforts have been made to find CB_2 selective agonists among the series and **4** is just one prominent example. There are also reports of CB_2 receptor agonists with monocyclic core structures. E.g., the tetrazine derivative **16** exhibited 90-fold selectivity for CB_2 and 2-imino-1,3-thiazolidine **17** showed more than 500-fold selectivity (Adam et al., 2006). A pyridone analogue **18** of the bicyclic CB_1 agonist CP47,597 showed CB_2 selective activity (CB_2 K_i = 56 nM) (Adam et al. 2006). Pyridine derivatives, such as **19** have been disclosed by Glaxo, and **19** was more than 100-fold selective for CB_2 over the CB_1 receptor (Adam et al., 2006).

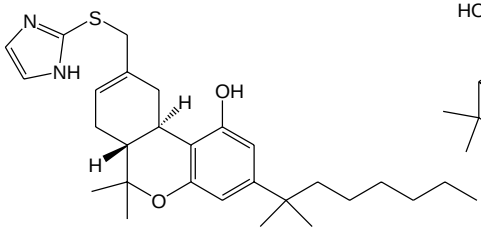
Several CB_2 antagonists have been reported (Griffin et al., 1999; Pertwee et al., 1995; Huffman, 2005). Although the Sanofi compound SR144528 (**20**) is the most widely used CB_2 antagonist/reverse agonist it does not always display typical antagonist behaviour (Rhee and Kim, 2002) and blocking experiments using this compound could thus be misleading. For example, we have recently reported on the unexpected strong anti-inflammatory effects of **20** (Raduner et al., 2006), which were probably not the result of an inverse agonist action at CB_2 receptors. Although less employed, the compound JTE-907 (**8**) is another CB_2 antagonist/reverse agonist (Iwamura et al., 2001; Ueda et al., 2005). The aminoalkylindole compound AM630 (**21**) appears to behave as a classical antagonist (52), but more studies are needed to show its utility to specifically block CB_2 signals without triggering unrelated signal transduction events. In spite of the number of CB_2-specific receptor ligands known (and their structural diversity), to the



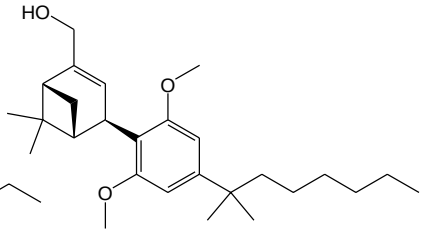
Acomplia™ (**1**)



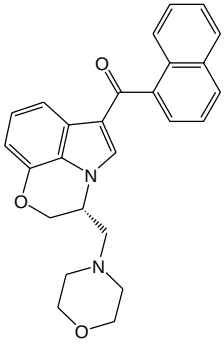
CP-55,940 (**2**)



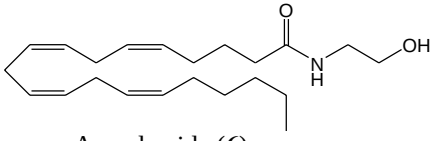
Cannabinor (**3**)



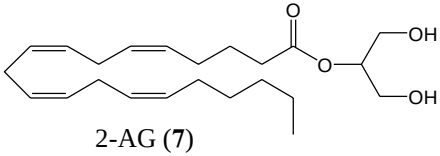
HU308 (**4**)



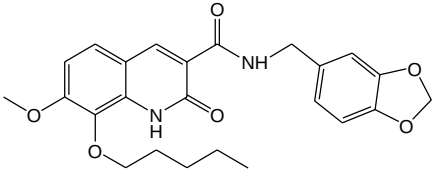
WIN 55,212-2 (**5**)



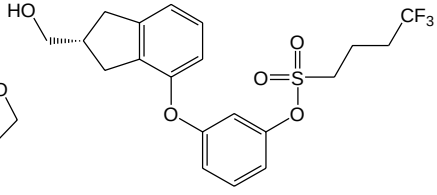
Anandamide (**6**)



2-AG (**7**)

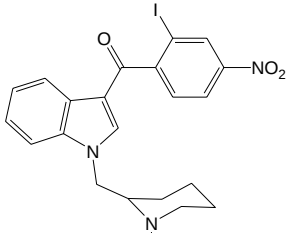


JTE907 (**8**)

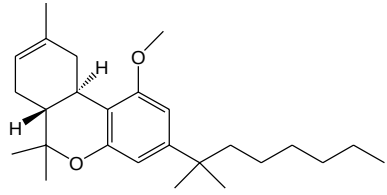


BAY 38-7271 (**9**)

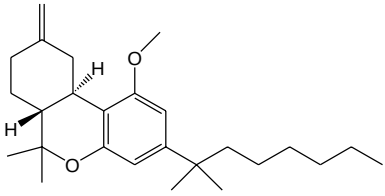
Fig. 4. Structures of CB receptor ligands discussed I.



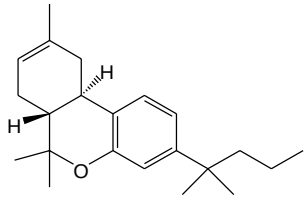
AM1241 (**10**)



L759633 (**11**)

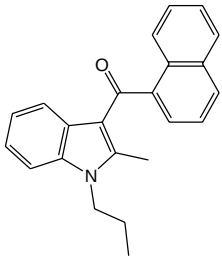


L759656 (**12**)

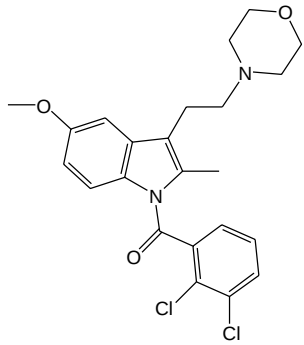


JWH-133 (**13**)

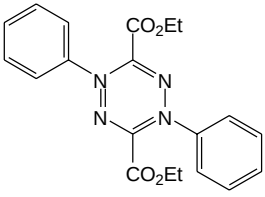
Fig. 4 continued. Structures of CB receptor ligands discussed I.



JWH-015 (**14**)



L768242 (**15**)



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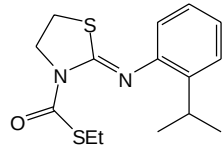

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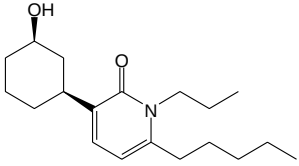
Fig. 5. Structures of CB receptor ligands discussed II.



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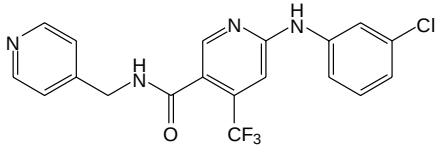

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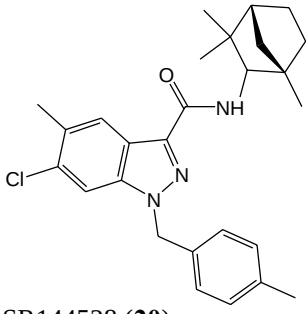
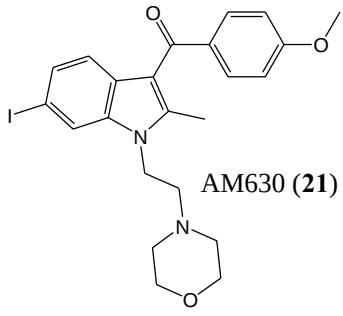
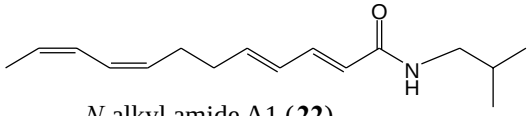


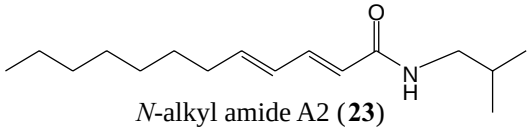
SR144528 (**20**)



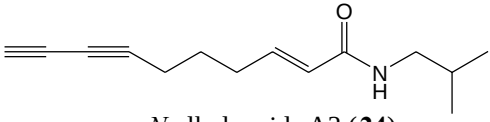
AM630 (**21**)



N-alkyl amide A1 (**22**)



N-alkyl amide A2 (**23**)



N-alkyl amide A3 (**24**)

Fig. 5 continued. Structures of CB receptor ligands discussed II.

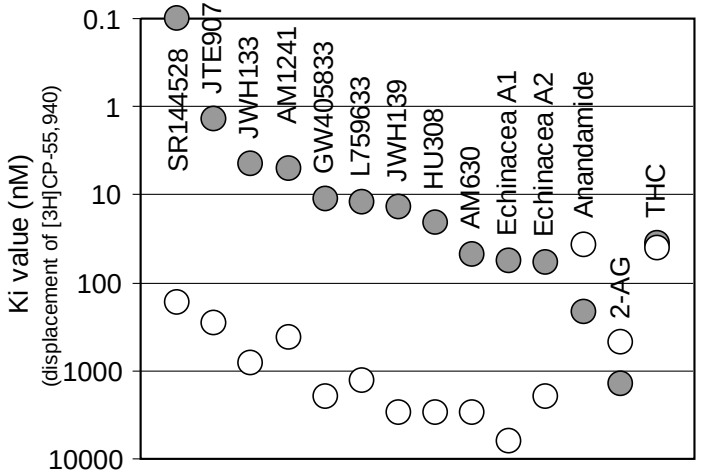


Fig. 6. Binding affinity and selectivity (CB_2 vs. CB_1) of different cannabinoid receptor ligands. White dots represent K_i-values for CB_1 receptors and the dark dots the K_i-values for CB_2 receptors. *Echinacea* compounds A1 (**22**) and A2 (**23**) show similar K_i-values as the CB_2 antagonist AM630 (**21**).

best of our knowledge, only the CB_2 receptor agonist **3** from Pharmos Ltd. has entered trials for clinical development (www.pharmoscorp.com), and Glenmark Pharmaceuticals has recently anounced clinical trial with a their CB_2-specific lead compound (www.glenmarkpharma.com).

Plant *N*-alkyl amides as new CB_2 cannabimimetics

Although not as widespread as other classes of natural products fatty *N*-alkyl amides are relatively abundant in plants and they have been found in a variety of families, including the Asteraceae, Brassicaceae, Leguminosae, Piperaceae, and Rutaceae (Chapman, 2004). These endogenous lipid constituents, which often occur in just trace concentrations, are believed to regulate a wide range of plant physiological processes, including growth and root development (Ramirez-Chavez et al., 2004). Moreover, natural fatty acid amides are often insecticidal (Molina-Torres et al., 2004) and their presence is thus likely to provide an evolutionary advantage. It has been shown that plants produce volatile fatty acid amides, such as linolenoyl-L-glutamine in response to elicitors in oral secretions of caterpillars (Lait et al., 2003). Other fatty acid *N*-alkyl amides frequently found in plants are *N*-acylethanolamines (derived from fatty acids 12:0; 14:0; 16:0, and 18:2) (Chapman et al., 1999), but despite their structural similarity to anandamide (ethanolamide head group) they have not been shown so far to possess affinities to cannabinoid receptors. Nevertheless, and although

anandamide itself has never been isolated from plant sources, it has been suggested that plant *N*-acylethanolamines modulate the endocannabinoid system in mammals by down-regulating FAAH, which leads to a longer half-life of the endogenous ligand anandamide (Di Marzo et al., 1998; Cravatt and Lichtman, 2003). We have recently investigated the pharmacological profile of extracts and individual constituents of the medicinal plant *Echinacea purpurea* and we were able to show that certain fatty acid *N*-isobutyl amides modulate TNF-α expression in monocytes via CB_2 receptors (Gertsch et al., 2004). These findings were confirmed in an independent study which demonstrated that the interaction of *N*-isobutyl amides and *N*-methylbutyl amides from *Echinacea angustifolia* with the endocannabinoid system in a rodent model (Woelkart et al., 2005). Together with our most recent observation that dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid *N*-isobutyl amide (**22**) and dodeca-2*E*,4*E*-dienoic acid *N*-isobutyl amide (**23**) from *Echinacea* directly and specifically interact with the human CB_2 receptor (K_i ~ 50 nM) and thereby elicit discrete functional responses (Raduner et al., 2006), these insights may lead to a revived interest in plant fatty acid amides other than *N*-acylethanolamines. Although *N*-alkyl amides from the medicinal plant taxon *Echinacea* were first reported as pharmacologically active entities already more than 10 years ago (Muller-Jakic et al., 1994), the discovery that members of this compound family bind to CB_2 receptors for the first time provides a mechanistic rationale for some of the pharmacological effects commonly associated with the medicinal plant *Echinacea*. In contrast to anandamide and 2-AG many plant *N*-alkyl amides produce a strong tingling effect, which is distinct from capsaicin and not directly related to the vanilloid receptor TRPV1 (Greger, 1988). The tingling of these compounds was shown to be associated to activation of tactile and thermal trigeminal neurons (Bryant and Mezine, 1999), but its underlying molecular mechanism remains unclear. **22** and **23** bind to CB_2 with ca. 30-50 fold selectivity over the CB_1 receptor (Raduner et al., 2006). In addition, binding to CB_2 was also shown to be highly selective relative to a large number of other receptors in a broad-based receptor screen, including 49 receptors of pharmacological relevance. Encouraged by these findings, we have now initiated the screening of different lipophilic plant extracts known to contain *N*-alkyl amides, in order to search for other CB_2 receptor binding compounds. E.g., *N*-alkyl amides similar to those of *Echinacea* can e.g. be found in *Spilanthes* and *Xanthoxylum* species. Rather satisfactorily, a number of different *N*-alkyl amide-rich plant extracts have alrady shown reproducible K_i values in the lower ng/ml range. As the structural diversity of naturally coccuring plant *N*-alkyl amides is limited and only few head groups are known in plants (Figure 7), we have also started to investigate synthetic analogs of these natural products. Preliminary data show that certain *N*-benzyl

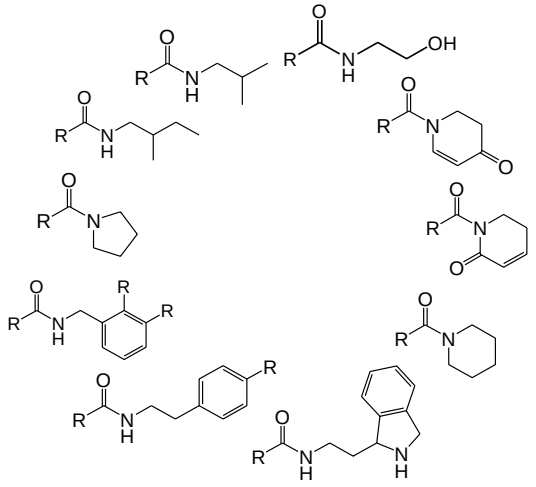


Fig. 7. Major plant derived *N*-amides which are found as head groups in fatty acid *N*-amides.

amides, which also naturally occur, have moderate affinity for CB_2 receptors if they are equipped with an apropriate acyl tail (unpublished data). In contrast to anandamide, *N*-alkyl amides, such as the ones from *Echinacea* are not metabolised by FAAH (Woelkart et al., 2005) and can be found in human plasma after oral ingestion with C_{max} values in the lower ng/mL range and a typical T_{max} of < 1h (Woelkart et al., 2005; Matthias et al., 2005). This difference in metabolic stability is likely a consequence of structural differences in the acyl tail. While the arachidonic acid part of anandamide leads to a very low metabolic stability *in vitro* and *in vivo* (Lin et al., 1998; Cravatt and Lichtman, 2003), *Echinacea* constituents are mainly derived from undeca and dodecanoic acid, with varying degrees of unsaturation and different double bond configurations in the acyl part. Although natural *N*-alkyl amides as such are not drug-like structures, e.g. they do not conform to the Lipinski rule of five (Lipinski et al., 2001), they may nonetheless have the potential to serve as novel scaffolds for the development of analogs with a better pharmacokinetic profile and/or as new tool compounds to study CB_2 receptor pharmacology.

The CB_2 receptor-bound conformation of *N*-alkyl amides

It has not been possible so far to determine the bioactive conformation of anandamide bound to the CB_2 receptor or even that of the ligand-free receptor by experimental methods. Attempts to determine the bioactive conformation of anandamide by computational methods using homology

models based on the structure of rhodopsin A proved to be difficult due to the high degree of flexibility of the anandamide arachidonic acid part, which contains four nonconjugated double bonds. In contrast, docking studies with **22** and **23** revealed a putative binding site in the CB_2 receptor, which is located adjacent to helices III, V, VI, and VII, near to the extracellular side of the 7TM bundles (Figure 8) (Raduner et al., 2006). According to this model the amide head group points into the hydrophilic pocket, which is formed by the side chains of Asp189 and Tyr190. The phenolic hydroxyl group of and the latter is engaged in a H-bond interaction with the amide hydrogen of **22** and **23**, while the phenyl ring itself is involved in π-π interactions with the C2-C3 double bond of the alkyl amide (Figure 9). Far less clear is the CB_2 receptor interaction of the acyl tail. Preliminary SAR data in our laboratory (Figure 10) show that

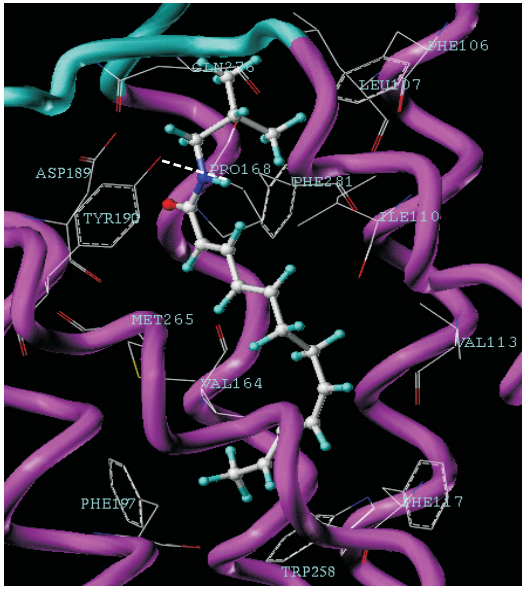


Fig. 8. Putative CB_2 receptor binding conformation of *N*-alkyl amides **22** and **23** based on a CB_2 homology model (Raduner et al., 2006).

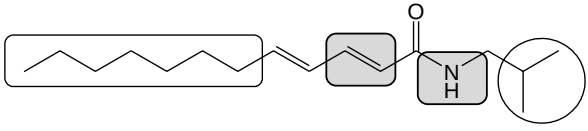


Fig. 9. *N*-isobutyl amide core structure showing the acyl tail and polar head (open boxes) and the central parts involved in CB_2 receptor binding-interaction (shadowed boxes).

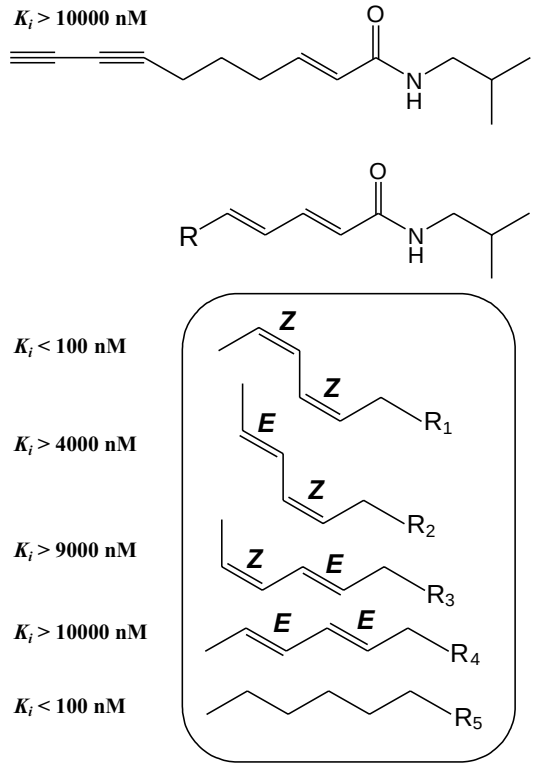


Fig. 10. Preliminary SAR of the acyl tail in *N*-isobutyl amides with regard to *h*CB_2 receptor affinity showing relative K_i-values.

configuration of the double bonds and the degree of flexibility of the acyl chain (Matovic et al., 2007) are important for binding, e.g. compound **24** does not bind to cannabinoid receptors (Raduner et al., 2006). Obvioulsy the above model can only be considered as putative and will have to be validated experimentally. This could e.g. involve investigation of the effects of mutations of Tyr190 in CB_2 on the binding of compounds **22** and **23**.

CB_2 receptor signal transduction

Although G_i/G_o mediated responses have been well established for the CB_1 receptor it has only recently been shown that CB_2 receptors also signal via G_o-proteins in monocytic cells. In fact, Sugiura et al. (2000) showed that the endocannabinoid 2-AG not only inhibits cAMP formation but also induces internal Ca^{2+} signals in a CB_2-dependent way. This CB_2-dependent stimulation of Ca^{2+} transients has been confirmed in our laboratory

(Raduner et al., 2006). Related studies have shown that the CB_2 receptor is linked to phospholipase C, possibly via G_o-proteins, and that anandamide can induce Ca^{2+} transients in pulmonary endothelial cells (Zoratti et al., 2003). In contrast to 2-AG and *N*-alkyl amides from *Echinacea*, the ethanolamine compound anandamide does not modulate intracellular Ca^{2+} transients in monocytes. As monocytes/macrophages are the relevant targets of CB_2 specific ligands this unexpected finding may also be relevant in a physiological setting. Moreover, interactions of the two endo-

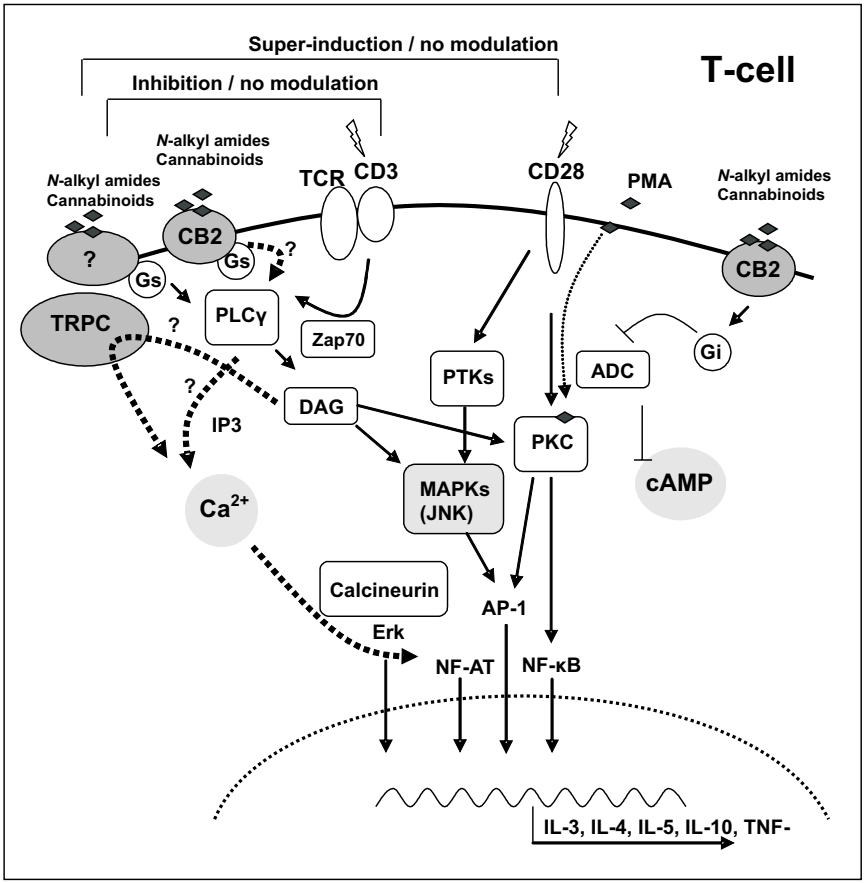


Fig. 11. Model of the molecular effects on *N*-alkyl amides and cannabinoids on the expression of cytokines in T-cells. The T_H1-T_H2 shift currently observed is (at least in the case of *N*-alkyl amides and anandamide) associated to differential modulation of CD3 and CD28 pathways, leading to increased IL-3, IL-4, IL-5 and IL-10 expression (Raduner et al., 2006). Modulation of Ca^{2+} transients by cannabinomimetics is not mediated by CB_2 receptors but TRAPC.

cannabinoids with the CB_2 receptor are likely to activate different signaling pathways by yet unknown mechanisms.

Whether cannabinoid ligand-induced Ca^{2+} transients in T-cells are also mediated via CB_2 receptors (Figure 11) remains debatable. It has been reported that Δ^9-THC and cannabinol robustly elevate Ca^{2+} in resting human and murine T cells in a CB_2-dependent way, whereas **2** does not influence Ca^{2+} transients (Rao et al., 2004). It may well be that in T-cells Ca^{2+} signaling is influenced by another yet unknown receptor. Recently, the Kaminski laboratory has proposed that classic tricyclic cannabinoids with structural similarity to Δ^9-THC elicit a robust influx of Ca^{2+} in T cells through receptor-operated cation channels in a manner sensitive to cannabinoid receptor antagonists, but independent of the CB_1 and CB_2 receptors (Rao and Kaminski, 2006). According to this hypothesis, the canonical transient receptor potential (CTRP) could be involved in this effect. Independent of these speculations, we believe that the endo-cannabinoids anandamide and 2-AG distinctly interact with cannabinoid receptors in distinctly different ways, despite the fact that they bind to the same or closely overlapping binding sites. As the *N*-alkyl amides **22** and **23** show the Ca^{2+} signaling effects of 2-AG and the antiinflammatory effects of anandamide, this class of natural products could also serve as model compounds to study CB_2 receptor interactions and signal transduction events.

Immunomodulatory effects of cannabinoids and *N*-alkyl amides from *Echinacea*

Anandamide and *N*-alkyl amides from *Echinacea* show potent effects on LPS-induced TNF-α expression in whole blood assays (Figure 12). Although LPS recognizing $CD14^+$ mononuclear cells strongly express the CB_2 receptor we found that the anti-inflammatory effect is not exclusively mediated via cannabinoid receptors (Raduner et al., 2006). Thus, anandamide inhibits the LPS-induced expression of pro-inflammatory cytokines IL-1β, IL-12 and TNF-β at low nanomolar concentrations (IC_{50} < 50 nM), in spite of its relatively moderate K_i value (hCB_2) of 218 ± 148 nM, suggesting that the effect could either be a consequence of low CB_2 receptor occupancy or could be mediated by another target. Based on data for **24**, which is structurally related to **22** and lacks CB_2 receptor affinity, but still shows the typical anti-inflammatory effects (Raduner et al., 2006), we currently favour the latter explanation. In contrast to IL-1β, IL-12 and TNF-α, the constitutive expression of IL-6 was up-regulated by CB_2 agonists in the same *ex vivo* whole blood model in a CB_2 receptor-dependent way, as indicated by competition experiments with the reverse antagonist SR144528 (Raduner et al., 2006). *N*-alkyl amides from *Echinacea* also

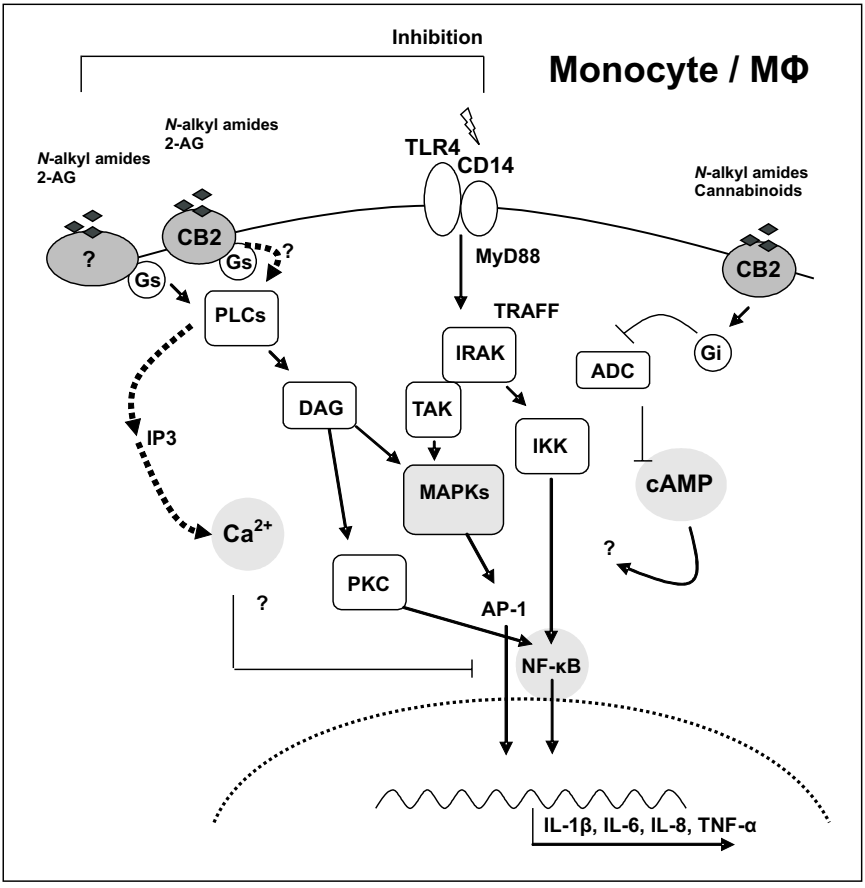


Fig. 12 Model of the intracellular effects of *N*-alkyl amides and 2-AG in monocytes/macrophages (Mϕ). The LPS stimuli mediated by CD14/TLR4 appear to be inhibited in a CB_2-independent way, but intracellular Ca^{2+} is increased by 2-AG and *N*-alkyl amides via CB_2 receptors.

inhibited LPS-induced activation of mouse RAW264.7 macrophages and thus exert anti-inflammatory effects (Chen et al., 2005). In studies with human subjects, lung aveolar macrophages removed from marijuana smokers were compromised in their ability to produce TNF-α, granulocyte/macrophage colony stimulation factor and IL-6 in response to LPS stimulation (Baldwin et al., 1997). It seems that cannabinoids can inhibit the production of TNF-β and other cytokines in several different models and by several different mechanisms, not all of which depend on interaction with cannabinoid receptors (for a review see Klein, 2005). While suppressing the production of cytokines, cannabinoids have been shown

to increase the production of cytokines (including TNF-α, IL-1, IL-6, and IL-10) when they are administered together with bacteria or antigens (Klein et al., 1993; Klein, 2005) or, in some cases, even when administered alone (Kishimoto et al., 2004). Therefore, cannabinoids *in vivo* could either suppress or enhance the production of these pro-inflammatory mediators, depending on either the nature of the stimulus or the type of cannabimimetic used.

N-alkyl amides from *Echinacea* have recently been shown to inhibit IL-2 expression in T helper-cells in the lower μM range (Sasagawa et al., 2006), which is an effect typical for cannabinoids (17). We have shown that *N*-alkyl amides from *Echinacea* at concentrations achievable upon oral administration in humans (lower nM range) have a T_H-biasing effect, in which T_H1-cell activity is suppressed and T_H2-cell activity is increased. The same effect has been shown for cannabinoids and this generally supports the potential use of cannabimimetics in the treatment of chronic inflammatory diseases. In the case of *N*-alkyl amides we could show that the T_H2 shift (i.e. the expression of the corresponding cytokines) is associated to superstimulation of CD28 and inhibition of CD3 sginalling (Figure 11). Overall, *N*-alkyl amides show similar immuno-modulatory effects as cannabinoids and therefore qualify to be classified as cannabimimetics.

Anti-inflammatory cannabinoids in diet—towards a better understanding of CB_2 receptor action?

The endocannabinoid system is an ancient lipid signalling network which in mammals modulates neuronal functions, inflammatory processes, and is involved in the aetiology of certain human lifestyle diseases, such as Crohn's disease, atherosclerosis, and osteoarthritis. The system is able to down-regulate stress-related signals that lead to chronic inflammation and certain types of pain, but it is also involved in causing inflammation-associated symptoms, depending on the physiological context. The cannabinoid type-2 (CB_2) receptor, which unlike the CB_1 receptor does not induce central side effects, has been shown to be a promising therapeutic target. While CB_1 receptor antagonists/inverse agonists are of therapeutic value, CB_2 receptor ligands including agonists are of pharmacological interest. Although the endocannabinoid system is known to be involved in the regulation of energy homoeostasis and metabolism (mainly via CB_1 receptors) there was hitherto no direct link between food intake and cannabinoid receptor activation. Our recent finding that beta-caryophyllene, a ubiquitous lipohilic plant natural product, selectively binds to the CB_2 receptor and acts as a full agonist is unexpected (Gertsch et al., 2008). Maybe even more unexpected is that oral administration of

this dietary compound exerts potent antiinflamamtory effects in wild type mice but not in CB_2 receptor ($Cnr2^{-/-}$) knockout mice. Like other CB_2 ligands also beta-caryophyllene inhibits the pathways triggered by activation of the toll-like receptor complex CD14/TLR4/MD2, which typically lead to the expression of proinflammatory cytokines (IL-1*b*, IL-6; IL-8 and TNF-alpha) and promotes a TH_1 immune response. Here, the CB_2 receptor-dependent effect of beta-caryophyllene on LPS-triggered activation of the kinases Erk1/2 and JNK1/2 are further discussed with respect to the possibility that both CB_2 inverse agonists and agonists, independent of their G-protein signalling, may block LPS-triggered activation of MAPKs, leading to inhibition of proinflammatory cytokine expression and attenuation of inflammation.

Apart from the well-known psychomimetic action of the classical cannabinoid Δ^9-tetrahydrocannabinol (THC) mediated by centrally expressed cannabinoid CB_1 receptors, it is now generally accepted that the endocannabinoid system also occupies regulatory roles in peripheral tissues (Di Marzo, 2008; Kunos and Osei-Hyiaman, 2008; Klein and Newton, 2007). The cannabinoid CB_2 receptor, which is involved in the tuning of inflammatory processes (Wolf and Ulrich, 2008; Wright et al., 2008; Lotersztajn et al., 2008) is able to mediate cellular signals that in many cases lead to attenuation of inflammation (*vide supra*). The endogenous CB receptor ligands, the arachidonic acid derivatives anandamide and 2-arachidonoylglycerol (2-AG), which are the major endocannabinoids known, partially or fully activate CB receptors in a rather non-selective manner and controlled by strict biosynthesis and degradation (Di Marzo, 2008).

Anandamide and 2-AG have been shown to inhibit the inflammatory processes triggered upon activation of the toll-like receptor complex CD14/TLR4/MD2 (i.e. LPS and carrageenan stimulation) (Klegeris et al., 2003; Gallily et al., 2000). The same effect has also been reported for other CB_2 receptor agonists like JWH133 (Duncan et al., 2008), HU-308 (Hanus et al., 1999), and *N*-alkylamides (Raduner et al., 2006). Paradoxically, also CB_2 receptor inverse agonists like JTE-907 (Ueda et al., 2005) SR144528 (Ueda et al., 2005; Oka et al., 2005), and Sch.336 (Lunn et al., 2006) show this effect in the same or similar models. As both CB_2 receptor inverse agonists (Lunn et al., 2008) and agonists block identical pathways in an apparently CB_2 receptor-dependent manner, mediators other than cAMP or calcium transients should be involved in this mechanism. In our study (Gertsch et al., 2008) treatment of primary monocytes/macrophages with the CB_2 receptor agonist beta-caryophyllene leads to inhibition of LPS-stimulated phosphorylation of the kinases Erk1/2 and JNK1/2 but not p38, despite the fact that the phosphorylation of these kinases is increased by this compound and JWH133 in resting (non-stimulated) monocytes/

macrophages. Thus, the context of differentially activated signalling pathways determines the way signals are integrated. Erk1/2 has been shown to be either activated or inhibited by different GPCRs, both via G-protein dependent and independent mechanisms, involving intracellular calcium transients (Chuderland et al., 2008) the b-arrestin scaffold (Gripentrog et al., 2008) and ubiquitination (Shenoy et al., 2005) JNK1/2 is a stress-activated protein kinase that can be modulated by GPCRs. Protein tyrosine kinases are thought to be involved in the regulation of signal transmission from GPCRs to activation of the JNK/SAPK kinase pathway (Dikic and Blaukat, 1999). To inhibit LPS-stimulated Erk1/2 and JNK phosphorylation (i.e. inhibition of their pathways) could be achieved by blocking MyD88, TRAF6 and IKK-γ, which would lead to reduced transcription of proinflammatory cytokines and metalloproteinases. The question remains how CB_2 receptor ligands as diverse as inverse agonists and full agonists do the same thing in an apparently receptor-dependent fashion and whether in all cases MAPKs are involved. A direct inhibition of NF-κB, which is the primary signal in LPS-induced gene expression, is more unlikely because this factor is inhibited only at higher μM-concentrations of cannabinoids. How exactly does the CB_2 receptor modulate MAPKs in immune cells? There are different possible mechanisms, such as hitherto neglected CB_2 pathways like the ras-MAPK pathway or even an indirect action via TNF-α expression. Interestingly, accumulation of TNF-α transcripts, but inhibition of protein expression has been shown after treatment with both CB_2 selective inverse agonists and agonists (Puffenbarger et al., 2000; Gertsch et al., 2004). On the same line, inhibition of the JNK pathway in monocytes/macrophages will inhibit TNF-α translation (Swantek et al., 1997). Moreover, TNF-α stimulated pathways could be inhibited indirectly, such that the pro-inflammatory feedback will shut down. In fact, there is good evidence that constitutive TNF-α is inhibited by the endocannabinoid system and that the CB_2 receptor is involved (Rajesh et al., 2008; Mechoulam et al., 2007). Moreover, TNF-α can activate the endocannabinoid system in adipose tissue (Kempf et al., 2007). As TNF-α triggers the activation of Ras, p38 MAPK, ERK1/2, SAPK/JNK and Akt pathways and ultimately pro-inflammatory cytokine expression and cellular proliferation and migration, it is also possible that the autocrine action of TNF-α may be inhibited by cannabinoids. The CB_2 agonists JWH-133 and HU-308 dose-dependently attenuate the effects of TNF-α (Batkai et al., 2007; Rajesh et al., 2007). On the other hand, activation of the CB_2 receptor by JWH133 in macrophages has been shown to activate Erk1/2 which leads to expression of IL-10 and thus a counteraction to proinflammatory gene expression (Correa et al., 2005). The CB_2 receptor has also been found to be a potential target to prevent atherosclerosis (Steffens et al., 2005), in part via TNF-α signalling

(Pacher et al., 2008). Because CB_2 receptor activation attenuates TNF-α-induced endothelial cell activation, transendothelial migration of monocytes, and monocyte/neutrophil-endothelial adhesion, and decreases TNF-α-induced proliferation and migration of human coronary vascular smooth muscle cells (Rajesh et al., 2007), in addition to inhibiting constitutive and LPS-stimulated TNF-α expression, the CB_2 receptor may be an essential pleiotropic modulator of TNF-α signal transduction. Providing that the multifunctional cytokine TNF-α is involved in numerous pathophysiological processes and the endocannabinoid system can dynamically regulate the levels and effects of this cytokine, more research should be dedicated to the molecular understanding of CB receptor signalling in the immune system.

Unfortunately, the pharmacological tool box for CB_2 receptors is still very limited, the results with selective agonists and antagonists often confusing and the conclusions drawn from such experiments potentially erroneous. For example, it would be wrong to say that 2-AG does mediate intracellular calcium in HL60 cells in a CB_2 receptor-independent manner because the selective CB_2 antagonist/inverse agonist AM630 cannot block this effect. The CB_2 inverse agonist SR144528 can exactly do this (Oka et al., 2005). In some experiments AM630 and SR144528 are able to block the anti-inflammatory effects of CB_2 agonists; in other experiments SR144528 is antiinflammatory. Thus, the classical concept of agonists and antagonists, "one lock and one key", does not sufficiently describe cannabinoid GPCR signalling. Even more puzzling is that CB_2 receptor-selective agonists like HU308 increase the inflammation caused by allergens in the skin, while CB_2 antagonists and CB_1 agonists inhibit inflammation (Karsak et al., 2007), thus pointing to the possibility that different cell types mediate distinct signals. The apparent involvement of CB receptors and Erk1/2 and JNK MAPKs in the production of inflammatory signals and skin cancer development after UVB irradiation seems to confirm this (Zheng et al., 2008).

Even though we do obviously not yet understand the underlying molecular mechanisms of these observations, it can be said that in most cases systemic administration of CB_2 receptor-selective ligands leads to attenuation of systemic inflammation in different animal models. It needs to be emphasized, however, that it is currently impossible to determine the molecular mechanism of a ligand *in vivo* and whether agonists are doing what antagonists should do and *vice versa*. One hint in that direction comes from data showing that the CB_1 antagonist SR141716A acts like a partial agonist *in vivo* (Smith et al., 2000).

In the case of the dietary natural product beta-caryophyllene, a full CB_2 receptor-selective agonist *in vitro*, potent antiinflammatory cannabimimetic effects are observed (Gertsch et al., 2008). Intriguingly, the lowest

oral dose tested (5 mg/kg) of this widespread and apparently non-toxic compound, which is also an FDA-approve food additive, was the most effective. Maybe this strengthens the hypothesis that beta-caryophyllene is indeed a dietary cannabinoid, thus inferring that by eating this compound the endocannabinoid system may be modulated in a beneficial way, possibly through modulation of TNF-α pathways, maybe even to such a degree that certain lifestyle diseases could be prevented. The finding that oral beta-caryophyllene is more effective in mice than JWH-133 despite its significantly lower CB_2 receptor affinity is encouraging. However, further studies will have to determine whether this natural product is also bioavailable in humans and in more general terms, how CB_2 receptor ligands exactly modulate inflammation *in vivo*.

Conclusions

Cannabis sativa and/or its constitutents show a variety of beneficial effects despite of the clear risk factor associated with CB_1 receptor activation. In particular, direct activation of CB_1 by Δ^9-THC may act pro-psychotic, possibly via increased dopamine signaling in the nucleus accumbes (Lupica and Riegel, 2005). Current data regarding the medicinal potential of cannabinoids and cannabimimetics thus suggest that CB_1 antagonists and CB_2 agonists are interesting pharmacophores. Even though at least three putative cannabinoid receptors independent of CB_1 and CB_2 are discussed (Mackie and Stella, 2006), the overwhelming data on CB_1 and CB_2-dependent pharmacological effects clearly show that these receptors are pharmacological targets. The recently postulated new cannabinoid receptor GPR55 may have physiological roles, such as modulation of pain reception (Staton et al., 2008), but too little is known to make a conclusion about the therapeutic potential of this receptor. CB_2 agonists have the potential to treat diseases related to chronic inflammation, pain, and bone formation, and CB_1 antagonists to metabolic disorders and potentially also liver cirrhosis. To date, a limited number of different cannabinoid ligands are available, most of which are derived from either Δ^9-THC (classical), CP55940 (non-classical), aminoakylindol (WIN55212) or eicosanoids (endogenous cannabinoids).

Natural products other than plant-derived cannabinoids comprise a hitherto neglected source for the discovery of potential new ligands for cannabinoid receptors. Morevoer, in light of the diversity of plant secondary metabolites traditional plant cannabinoids may not be the only class of natural products with affinities to cannabinoid receptors. Our discovery that plant *N*-alkyl amides and beta-caryophyllene exert CB_2-specific cannabimimetic effects clearly confirms this assumption. While potent bioavailable CB_1 agonists, like Δ^9-THC, may have been discovered during

the course of human experimentation with psychoactive plants, agonists directed at CB_2 and CB_1 antagonists may have remained undetected. The discovery of new natural non-cannabinoid cannabimimetics could therefore contribute to the development of more effective drugs for distinct diseases. Natural product chemists have thus a unique opportunity to participate in the existing cannabinoid-receptor directed drug discovery and find new natural products which bind to and either activate or block cannabinoid receptors. This is best summarized by a recent quote by Vincenzo Di Marzo (2007). 'Thus, the exciting promenade from plant natural products to animal physiology and back might soon become a true treasure hunt.'

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12 Anti-inflammatory Herbal Medicines for the Control of Pain

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Introduction

About 60 to 70% of the patients suffering from painful osteoarthritis or low back pain may benefit from herbal medicines. The advantage of treatment with herbal medicines over synthetic pain killers is the broader mechanism of action (Table 1a and 1b) and of lower risk for adverse events (see below).

The active principle of herbal medicines

Herbal medicine contains many co-active compounds. The effect of a herbal medicine is the sum of the action of all the active compounds which is

called 'the active principle'. When the combined effect of a mixture of compounds is equal or greater than the sum of individual effects, the phenomenon is called synergism. However, if the overall effect is less than that predicted from individual compounds, the phenomenon is called 'antagonism'. The active principle of the herb is concentrated in the preparation by solvent extraction from various parts of the plants including roots, leaves, flowers and fruits. Polar solvent is used to extract primarily hydrophilic compounds and nonpolar solvent for lipophilic compounds. Aqueous and alcoholic extracts (high and medium polarity) differ therefore in their profile of action, so that the results of clinical studies obtained with aqueous extracts cannot be transferred to extracts prepared with other solvents except their active principle has been demonstrated to be essentially similar (Chrubasik and Roufogalis, 2003). For example, the active principles of *Harpagophytum procumbens* extracts prepared with water or 60% ethanol differ considerably. The ethanolic extract contains—for technical reasons—at the most 50% of the harpagoside content of the aqueous extract Doloteffin^{R} (Sporer and Chrubasik, 1999).

Targets for the active principle of herbal medicines

The mechanisms of inflammation and cartilage destruction are shown in Figure 1. The inflammatory pathways include arachidonic acid cascades, vascular permeation induced by nitric oxide (formed from arginine catalysed by inducible nitric oxide synthase (iNOS) and cytokines acting as chemotaxis agents and by activation of COX-2 enzyme. Cartilage destruction is largely a result of protein matrix hydrolysis by the proteolytic enzymes including elastase, hyaluronidase and matrix-metalloproteinases (MMPs). MMP activity is inhibited by MMP inhibitors including tissue inhibitor of metalloproteinase (TIMP). Cartilage destruction is also induced by cytokines and oxygen radicals.

Arachidonic acid is released by hydrolysis of the cell membrane lipid by phospholipase A2 at the site of injury and converted to the inflammatory mediators leukotrienes (LTs) by lipoxygenase enzymes (LOXs), thromboxanes (TXs) and prostaglandins (PGs) by the cyclooxygenase enzymes (COXs). Linoleic acid (LA) and γ-linolenic acid (GLA) which belong to the ω-6 fatty acid family are the dietary source of arachidonic acid. α-Linolenic acid, stearidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) belong to the ω-3 fatty acid family. The biosynthesis of leukotrienes (LTs), thromboxanes (TXs) and prostaglandins (PGs) from the ω-3 and ω-6 fatty acid family utilizes the same series of enzymes (not shown).

Herbal medications interact to various extents with the inflammatory

Table 1a. Effect mechanism suggested from *in vitro* studies (GLA gamma-linolenic acid)

	Inhibition of					*Antioxidative effect*
	COX-1	*COX-2*	*LOX*	*Cytokines*	*Elastase* Hyaluronidase*	
Avocado soybean ***Persea americana Glycine max***	Not investigated	Henrotin et al., 2003	Not investigated	Mauviel et al., 1991; Kut-Lasserre et al., 2001; Henrotin et al., 2003; Andriamanalijaona et al., 2006	Kut et al., 1998	Kim et al., 2000; Au et al., 2007
Blackcurrent ***Ribes nigrum***	Pham et al., 1986; Garbacki et al., 2002	Not investigated Not investigated	Not investigated	Garbacki et al., 2005	Not investigated	Costantino et al., 1993
Cat's claw** ***Uncaria* species**	Aguilar et al., 2002	Aguilar et al., 2002	Not investigated	Sandoval et al., 2000, 2002; Allen-Hall et al., 2007; Miller et al., 2006; Aguilar et al., 2002	Not investigated	Sandoval et al., 2000; Sandoval et al., 2002; Goncalves et al., 2005; Pilarski, 2006
Devil's claw* ***Harpagophytum procumbens***	No activity Fiebich et al., 2001	Fiebich et al., 2001; Chrubasik et al., 2002; Kundu et al., 2005;	Loew et al., 2001	Fiebich et al., 2001; Chrubasik et al., 2002; Huang et al., 2006; Chrubasik et al., 2006	*Boje et al., 2003	Kaszkin et al., 2004; Huang et al., 2006

Contd.

Table 1a continued

	Inhibition of					*Antioxidative effect*
	COX-1	*COX-2*	*LOX*	*Cytokines*	*Elastase* Hyaluronidase*	
		Na et al., 2004; Huang et al., 2006				
Ginger ***Zingiber officinalis***	Wu et al., 1993 Nurtjahja-Tjendraputra et al., 2003	Tjendraputra et al., 2001 Frondoza et al., 2004; Kim et al., 2004; Kiuchi et al., 1992	Kiuchi et al., 1992	Frondoza et al., 2004; Kim et al., 2004	*Tsukahara et al., 2006	Wang et al., 2003; Kuo et al., 2005; Asnani et al., 2007; Cao et al., 1993
Nettle herb	El Haouari et al., 2006; Obertreis et al., 1996(a)	Not investigated	Obertreis et al., 1996(a)	Obertreis et al., 1996(b); Teucher et al., 1996; Riehemann et al., 1999; Klingelhöfer et al., 1999; Schulze-Tanzil et al., 2002	Melzig et al., 2001	Ozen et al., 2003; Kanter et al., 2005; Harput et al., 2005
Rose hip and seed ***Rosa canina***	Jäger et al., 2007; Wenzig et al., 2007	Jäger et al., 2007	No activity Wenzig et al., 2007	Not investigated	Rennert et al., 2002	Wenzig et al., 2007

Contd.

Table 1a continued

	Inhibition of					*Antioxidative effect*
	COX-1	*COX-2*	*LOX*	*Cytokines*	*Elastase* Hyaluronidase*	
Salai guggal ***Boswellia serrata***	Siemoneit et al., 2007	No activity Ammon et al., 1993; Siemoneit et al., 2007	Ammon et al., 1991, 1993; Poeckel et al., 2006	Roy et al., 2005, 2006; Gayathri et al., 2007; Takada et al., 2006	Safayhi et al., 1997	Pro-oxidative Altman et al., 2004; Glaser et al., 1999
Seed oils with GLA ***Borago officinalis*** ***Oenothera biennis*** ***Ribes nigrum***	Iverson et al., 1992	Not investigated	Iverson et al., 1992	DeLuca et al., 1999; Furse et al., 2001, 2002; Dooper et al., 2003	Jiang et al., 1996	Not investigated
Willow bark ***Salix* species**	Khayyal et al., 2005	Krivoy et al., 2001; Fiebich & Chrubasik, 2004; Khayyal et al., 2005	Wurm et al., 1982; Khayyal et al., 2005	Fiebich and Chrubasik, 2004; Khayyal et al., 2005	Kuppsamy et al., 1990	Kahkonen et al., 1999; Rohnert et al., 1998; Khayyal et al., 2005

*anticonvulsive action (Mahomed and Ojewole, 2006).
**interaction with 5-HT2 receptors (Jürgensen et al., 2005).

cascade (COX-1 and/or COX-2 and/or LOX) (Table 1a), but experimental data also indicate interaction with the production of cytokines, the mediators of cartilage destruction (Table 1a and b). Other mechanisms of action include elastase or hyaluronidase inhibition, antioxidative effectiveness (Table 1a) and other still unidentified effects that may contribute to the overall analgesic and joint protective effects.

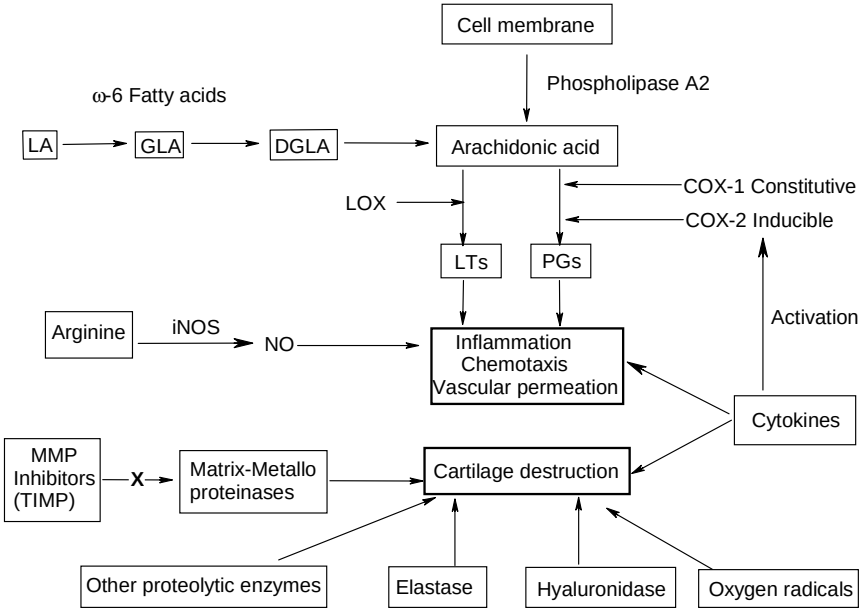


Fig. 1. Inflammatory targets for herbal medicinal products. The scheme illustrates the mechanism for inflammation induced by various agents including leukotrienes and prostaglandins from arachidonic acid cascade, matrix-metalloproteinases, nitric oxide (NO) from oxidation of arginine catalysed by iNOS (inducible nitric oxide synthase) and cytokines, and for the cartilage destruction induced by oxygen radicals, elastase, hyaluronidase, other proteolytic enzymes and cytokines. Linoleic acid (LA) and γ-linolenic (GLA) are consumed with nutrition.

Classification and effectiveness of herbal medicines in clinical use

We classify the anti-inflammatory herbal medicines as follows: (1) self-treatment according to traditional use, (2) food supplements and, (3) medicinal products. Their evidence of effectiveness has recently been summarized in a systematic review (Chrubasik et al., 2007a). Currently, there are not sufficient safety data of anti-inflammatory herbal medicines

Table 1b. Interaction of herbal medicines with cytokine release

	Inhibition of Cytpokines				
	Interleukin-1ß	*TNF-α*	*NF-κB*	*MMPs*	*Others*
Avocado soybean ***Persea americana Glycine max***	Mauviel et al., 1991; Henrotin et al., 2003; Andriamanalijaona et al., 2006; Au et al., 2007	Au et al., 2007	Gabay et al., 2007; Gabay et al., 2007	Henrotin et al., 1998; Gabay et al., 2007	Henrotin et al., 1998, 2003 2006; Altinel et al., 2007; Boumediene et al., 1999; Andriamanalijaona et al., 2006
Blackcurrent ***Ribes nigrum***	Not investigated	Not investigated	Not investigated	Not investigated	Garbacki et al., 2005
Cat's claw** ***Uncaria* species**	Allen-Hall et al., 2007	Sandoval et al., 2000; Sandoval et al., 2002; Allen-Hall et al., 2007	Aguilar et al., 2002	Not investigated	Miller et al., 2006
Devil's claw* ***Harpagophytum procumbens***	Chrubasik et al., 2002	Fiebich et al., 2001; Chrubasik et al., 2002	Huang et al., 2006	Not investigated	Not investigated
Ginger ***Zingiber officinalis***	Not investigated	Frondoza et al., 2004; Lantz et al., 2007	Frondoza et al., 2004	Not investigated	Not investigated

Contd.

Table 1b continued

	Inhibition of Cytpokines				
	Interleukin-1ß	*TNF-α*	*NF-κB*	*MMPs*	*Others*
Nettle herb	Obertreis et al., 1996(b); Teucher et al., 1996	Obertreis et al., 1996(b); Teucher et al., 1996	Riehemann et al., 1999	Schulze-Tanzil et al., 2002	Riehemann et al., 1999; Klingelhoefer et al., 1999
Rose hip and seed ***Rosa canina***	Not investigated	Not investigated	Not investigated	Not investigated	Not investigated
Salai guggal ***Boswellia serrata***	Gayathri et al., 2007	Gayathri et al., 2007	Takada et al., 2006	Roy et al., 2006	Roy et al., 2005; Gayathri et al., 2007
Seed oils with GLA	Furse et al., 2001, 2002; DeLuca et al., 1999; Dooper et al., 2003	DeLuca et al., 1999; Dooper et al., 2003; Furse et al., 2001	not investigated	not investigated	Not investigated
Willow bark ***Salix* species**	Fiebich & Chrubasik, 2004; Khayyal et al., 2005	Not investigated	Not investigated	Not investigated	Khayyal et al., 2005

TNF—tumor necrosis factor; NF—nuclear factor; MMPs—metalloproteinases; Others—other cytokines.

available to recommend them during pregnancy or lactation. Externally used anti-inflammatory herbal medicines are not considered in this chapter.

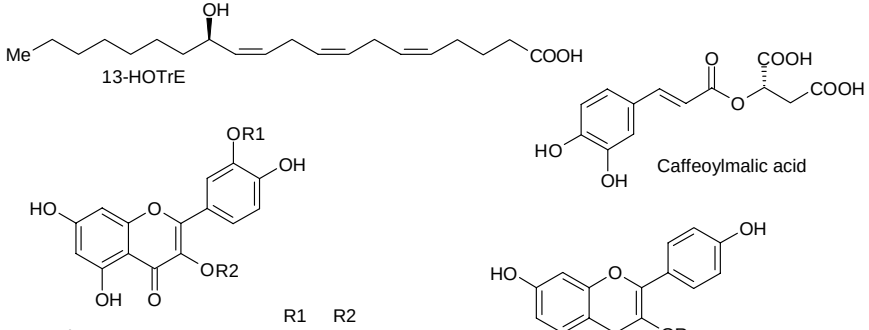
Anti-inflammatory herbal medicines for self-treatment according to traditional use

The cost of self-treatment with anti-inflammatory herbal medicines is low compared with synthetic anti-inflammatory drugs. Nettle (*Urtica dioica*) and blackcurrant leaf (*Ribes nigrum*) are listed in the European Scientific Cooperative on Phytotherapy (ESCOP) monographs (N.N., 2003). From our own experience, we recommend that ginger (*Zingiberis officinalis*) is added to the list.

Urtica dioica L.

The active principle of the herb or leaf of *Urtica dioica* (herba or folium *Urticae dioicae*) contains a monohydroxy polyunsaturated fatty acid (13-HOTrE), phenylcarbonic acids (e.g. caffeoylmalic acid, flavonoids (principally kaempferol, isorhamnetin, quercetin and their 3-rutinosides and 3-glucosides) (Figure 2) and other compounds not yet identified.

The active principle of nettle herb interacts with the arachidonic acid metabolism but also with the release of cytokines (Table 1a and 1b). A proprietary nettle extract as well as 13-HOTrE suppressed *in vitro* the expression of several matrix metalloproteinases (Schulze-Tanzil et al., 2002)



	R1	R2
Quercetin	H	H
Quercetin 3-*O*-rutinoside	H	β-D-rutinosyl
Quercetin 3-*O*-β-D-glucoside	H	β-D-glucosyl
Isorhamnetin	Me	H
Isorhamnetin 3-*O*-rutinoside	Me	β-D-rutinosyl
Isorhamnetin 3-*O*-β-D-glucoside	Me	β-D-glucosyl

	R
Kaempferol	H
Kaempferol 3-*O*-rutinoside	β-D-rutinosyl
Kaempferol 3-*O*-β-D-glucoside	β-D-glucosyl

Fig. 2. The structures of the active principle 13-HOTrE and co-active compounds in nettle herb or leaf.

known to be involved in cartilage destruction. The ESCOP monograph recommends nettle herb as adjuvant in the symptomatic treatment of osteoarthritis, rheumatoid arthritis, and/or rheumatic conditions (Anonymous, 2003). For internal use in adults, 15 ml of fresh juice up to three times daily is recommended, but from our own observations, this dose was not effective in the treatment of pain.

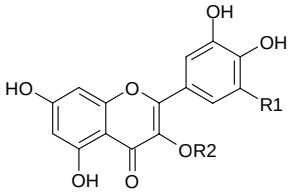
A pilot study indicated that 50 g of a nettle leaf stew (heated up to not more than 80°C) helped to save on additional consumption of diclofenac in patients suffering from acute knee osteoarthritis (Chrubasik et al., 1997). Tea (aqueous extract) was ineffective in alleviating osteoarthritic pain or pain in the lower back (own observation). *Urtica urens* may also be of help but contains a lower quantity of co-active ingredients (flavonoids, phenylcarbonic acids). In Germany, there are commercially available proprietary lipophilic nettle extracts, but their clinical effectiveness have not been demonstrated so far.

It is recommended that the plastic gloves are worn when harvesting and handling nettle leaves. After careful washing, the leaves should be cut into small pieces, then mixed with any hot vegetable at the end of the cooking time. Specific adverse events include allergy in few subjects (Anonymous, 2003) and occasionally minor gastrointestinal complaints.

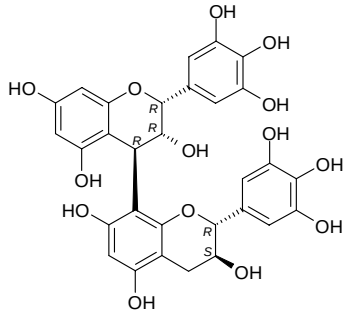
Ribes nigrum L.

The ESCOP monograph (Anonymous, 2003) recommends the leaf of *Ribes nigrum* (ribis nigri folium) as adjuvant in the treatment of rheumatic conditions. Characteristic compounds of the active principle in blackcurrant leaf include mono- and diglycosides of quercetin and kaempferol, mainly isoquercitrin and rutin, monomeric flavanols (mainly gallocatechin and epigallocatechin), proanthocyanidins and hydroxycinnamic acid derivatives (Figure 3). Moreover, unsaturated fatty acids (Dobson et al., 2002) and proanthocyanidins belong to the active principle.

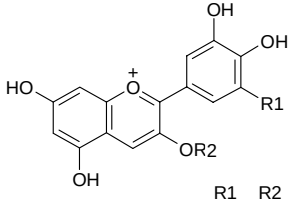
While interactions with the arachidonic acid cascade, cytokines and antioxidative activity have been demonstrated (Table 1a and 1b), interaction with other targets (Figure 1) remains to be shown. The dried leaves should be consumed as an infusion (20-50 g/litre infused for 15 minutes), 250-500 ml daily. Due to its diuretic action, blackcurrant leaf should not be taken concurrently with diuretics indicated for cardiac or renal insufficiency except on medical advice. There are no clinical data available on the anti-inflammatory effect of blackcurrant leaf, however animal experiments indicate that *Ribes nigrum* acts as anti-inflammatory drug (Declume, 1989).



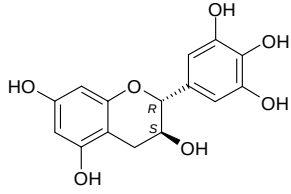
	R1	R2
Quercitin 3-*O*-β-D-glucofuranoside	H	β-D-glucofuranosyl
Quercitin 3-*O*-β-D-rutinoside	H	β-D-rutinosyl
Myricitin 3-*O*-β-D-glucofuranoside	OH	β-D-glucofuranosyl



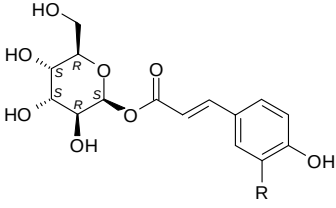
Proanthocyanidin



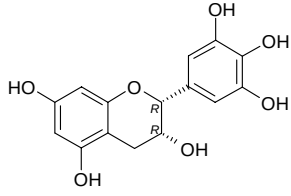
	R1	R2
Cyanidin 3-*O*-β-D-glucopyranoside	H	β-D-glucopyranosyl
Cyanidin 3-*O*-β-D-rutinoside	H	β-D-rutinosyl
Delphinidin 3-*O*-β-D-glucopyranoside	H	β-D-glucopyranosyl
Delphinidin 3-*O*-β-D-rutinoside	H	β-D-rutinosyl



Gallocatechin



	R
1-*O*-(E)-*p*-Coumaroyl-β-D-Glucopyranose	H
1-*O*-(E)-Caffeoyl-β-D-glucopyranose	OH
1-*O*-(E)-Feruloyl-β-D-glucopyranose	OMe



Epigallocatechin

Fig. 3. Structures of co-active compounds of the active principle in blackcurrant leaf.

Zingiber officinalis L.

There is no doubt that ginger contains anti-inflammatory active principle (Chrubasik et al., 2005a). Crude ginger contains up to 9% lipids or glycolipids largely made up with linoleic acid (Figure 4) which would contribute to the anti-inflammatory effect, and about 5 to 8% oleoresin.

The pungent principle, accounting for 25% of the oleoresin, consists mainly of gingerols. [6]-gingerol (the main gingerol) is more pungent than [8]-gingerol or [10]-gingerol (Figure 4) (Chrubasik et al., 2005a). Gingerols

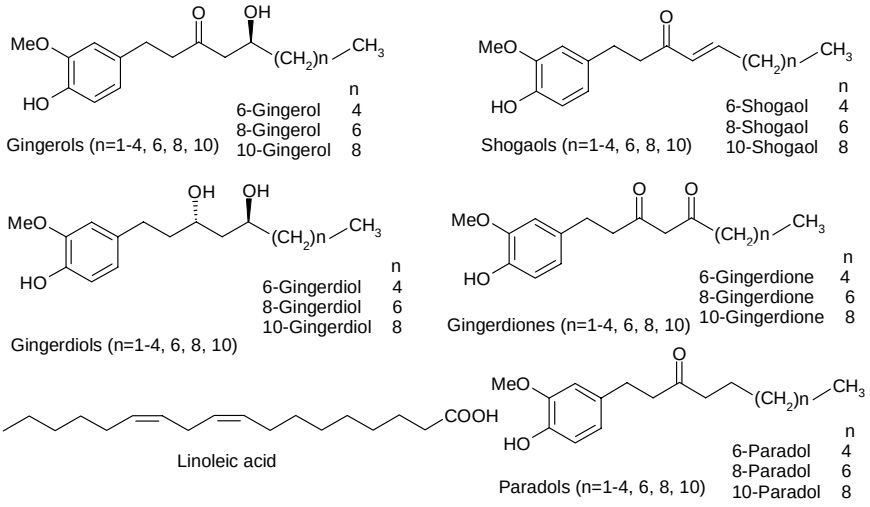


Fig. 4. The structures of gingerols—co-active compounds of the active principle of ginger.

together with the minor constituents including the corresponding ketones (gingerdiones), alcohols (gingerdiols) and deoxygenated products (paradols) contribute to the active principle in fresh ginger, and also the corresponding dehydrated product (shogaol) to the active principle in the dried rhizome (Figure 4).

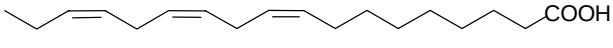
Raw ginger (zingiberis rhizoma) squeezed and added to fruit juice may alleviate a headache or other pain (own observation). The pungent principle in ginger may produce gastric pain after oral intake of ginger preparations. Specific adverse events have not yet been described during use of ginger as a pain-relieving herbal medicine. However, the anti-inflammatory clinical effectiveness of ginger has not yet been assessed systematically. For some extracts, not commercially available in Europe, moderate effectiveness in the treatment of osteoarthritc pain has been demonstrated (Chrubasik et al., 2007a).

Food supplements

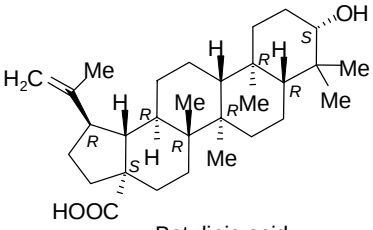
Food supplements are commercial herbal medicinal products with no claim for health benefit. To claim benefit in the treatment of osteoarthritis or low back pain, these medicines need to undergo governmental regulations that are associated with high cost. However, there is clinical evidence available, that a powder from rose hip and seed, and γ-linolenic acid containing seed-oils (see below) are of help in saving on the consumption of synthetic anti-inflammatory drugs.

Rosa canina L. subspecies *lito*

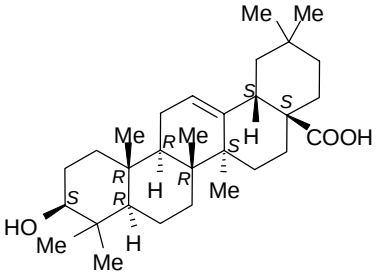
A systematic review on a powder from rose hip and seed (rosae pseudofructus cum fructibus) found moderate evidence of effectiveness in the treatment of painful osteoarthritis. Two clinical studies of good quality showed superiority of the proprietary product Litozin^{R} over placebo (Chrubasik et al., 2006a). Recently, a randomized double-blind study indicated that this powder is also effective in rheumatoid arthritis



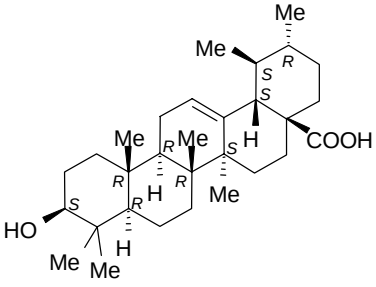
α-Linolenic acid



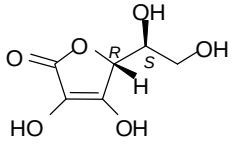
Betulinic acid



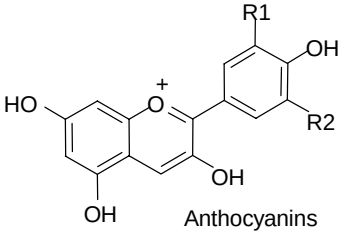
Oleanolic acid



Ursolic acid



Ascorbic acid



Anthocyanins

	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OMe	H
Delphinidin	OH	OH
Petunidin	OMe	OH
Malvidin	Me	Me

Fig. 5. Structures of co-active compounds of the anti-inflammatory active principle in rose hip.

(Rossnagel et al., 2007). A survey was undertaken to find out whether the powder is effective in the treatment of low back pain and the results were surprising as 60% of the patients were responders (Chrubasik et al., in press). The anti-inflammatory active principle is lipophilic and contains a galactolipid, linoleic acid (Figure 4) and α-linolenic acid and, triterpene acids including betulinic, oleanolic and ursolic acids, ascorbic acid, flavonoids and anthocyanins (Figure 5). Interaction with COX-1 and COX-2 has recently been demonstrated (Jäger et al., 2007). In contrast to the extract prepared from rose hip peels (rosae pseudofructus sine fructibus), a proprietary powder from rose hip and seed had no impact on lipoxygenase. Extracts from both, rose hip and rose hip and seed exerted anti-oxidative activity (Wenzig et al., 2007).

Similar to the aqueous extract from *Harpagophytum procumbens*, the treatment of powder from rose hip and seed may require up to 3 to 4 months until the maximum analgesic effect is achieved. Depending on the intensity of the complaints, up to 10 g powder per day may be required. Occasional allergy was observed as sole specific adverse event. Some patients may suffer minor gastrointestinal adverse effect. In case of constipation, 300 to 500 ml water or other liquids should be consumed when taking the powder. The volume of plant fibres increases by absorption of the liquid and may act as a laxative (like other plant fibres such as linseed (*Linum ustitatissimum*) or psyllium (*Plantago psyllia*). In case of irritable bowel syndrome with diarrhea, the plant fibres should not be taken with a large amount of liquid. A 2-hours gap between intake of the powder and other medications is recommended, to minimize the risk of interaction in the absorption of concomitant medications.

γ-Linolenic acid containing seed oil

The seed oil of *Oenothera biennis* L., *Borago officinalis* L. and *Ribes nigrum* L. contains γ-linolenic acid (Figure 6), which interacts with the mediators of inflammation and cartilage destruction (Table 1a and 1b). However, up to 3 g seed oil per day is required for an impact on rheumatic complaints (Ernst and Chrubasik, 2000). Long-term studies are urgently required that prove clinical effectiveness in the treatment of painful osteoarthritis and low back pain.

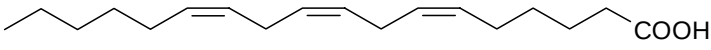


Fig. 6. The structure of γ-linolenic acid.

Herbal medicinal products

a) Preparations of Harpagophytum procumbens (Burch.) DC.

More than 20 studies investigated the effectiveness of preparations of the secondary roots of *Harpagophytum procumbens (*Burch.*)* DC. (harpagophyti radix). The ESCOP monograph (N.N., 2003) recommends devil's claw as symptomatic treatment of painful osteoarthritis and relief of low back pain. The daily dose should be based on up to 9 g crude drug, equivalent to up to 100 mg of harpagoside (Figure 7). Other compounds of the active principle include harpagide, procumbide and phenolic glycosides (Figure 7).

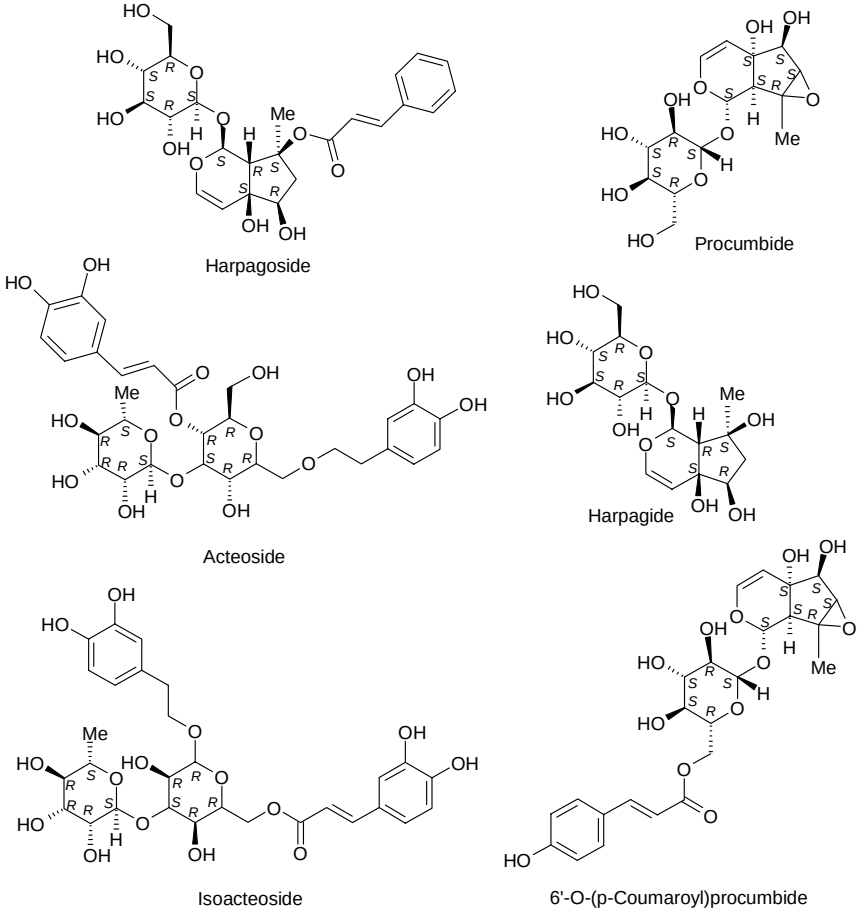


Fig. 7. Structures of co-active compounds of the active principle of *Harpagophytum procumbens.*

Although the active principle has not yet been characterized in detail, a broad mechanism of action has been demonstrated including interaction with the arachidonic acid cascade and cytokine release (Table 1a and 1b).

Duration of treatment should last at least 2-3 months. If symptoms persist, medical advice should be sought. Evidence of clinical effectiveness of *Harpagophytum procumbens* in the treatment of osteoarthritis and/or low back pain has been summarized in six systematic reviews (Chrubasik et al., 2007a). A confirmatory study supported by many exploratory studies of good quality demonstrated that aqueous extract with more than 50 mg harpagoside in the daily dosage (e.g. Doloteffin^{R}) is effective in alleviating musculoskeletal pain. Likewise, a confirmatory and several exploratory studies demonstrated effectiveness of the cryoground powder Harpadol^{R} (daily supply of harpagoside 60 mg/day) in the treatment of osteoarthritic pain.

However, studies with a confirmatory design were not able to demonstrate clinical effectiveness of an extract prepared with 60% ethanol (daily supply of harpagoside up to 30 mg/day).

Dose-dependent effectiveness was shown for aqueous extract: the larger the dose, the greater the effectiveness. In addition, onset of full effectiveness may require up to 3 to 4 months administration as suggested by the ESCOP monograph (N.N., 2003) and also shown in two surveys over one year duration (Chrubasik et al., 2005b, 2007b). Sensitive people may suffer from gastrointestinal complaints due to the bitter principle in devil's claw—the co-active iridoid glycosides (Figure 7). A systematic review on the adverse events indicated that the *Harpagophytum* medicinal products used in the dose recommended by the ESCOP monograph are well tolerated (Vlachojannis et al., 2008). In addition, a study has shown an increase in gene expression of tissue inhibitor of metalloproteinases in a canine osteoarthritis model for *Harpagophytum* extract suggesting the likelihood of possible cartilage-damage-preventative effect (Chrubasik et al., 2006b).

b) Preparations from Salix species L.

Willow bark (salicis cortex) consists of the whole or fragmented dried bark of young branches or whole dried pieces of current year twigs of various species of the genus *Salix* including *S. purpurea*, *S. daphnoides* and *S. fragilis*. The drug contains not less than 1.5% of total salicylic derivatives, expressed as salicin (Figure 8).

It is generally thought that salicin metabolites are the main co-active compound of the active principle in willow bark. However, a daily dose of willow bark extract providing 240 mg of salicin corresponds only to a dose of about 100 mg of salicylic acid. Therefore other compounds are mainly

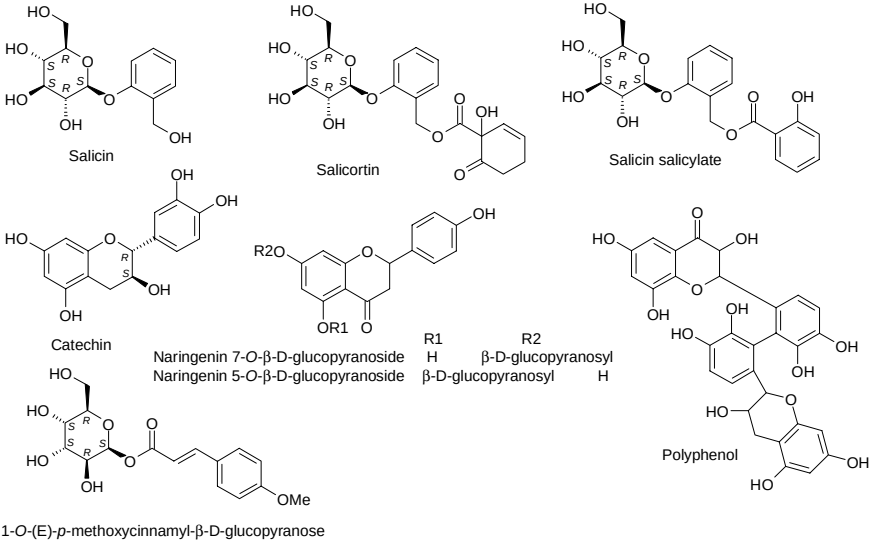


Fig. 8. Co-active compounds of the active principle of *Salix* species.

responsible to explain the potent anti-inflammatory and analgesic effects of willow bark (Schmid, 1998), e.g. flavonoids (naringenin and other glucosides), flavanols (catechin), polyphenols and hydroxycinnamic acid derivatives including *p*-methyoxycinnamyl, *p*-coumaroyl, feruloyl glucosides (Figures 3 and 8). It remains to be established which compounds contribute to the willow bark broad mechanism of action (Table 1a and 1b).

The ESCOP monograph (N.N., 2003) recommends willow bark for the relief of low back pain, symptomatic relief of mild osteoarthritic and rheumatic complaints. The daily dose should be based on the intake of up to 240 mg of total salicin. In case of sensitivity of salicylates, intake of willow bark preparations should be avoided.

The ethanolic extract Assalix^R with 120 bis 240 mg salicin in the daily dosage was investigated in several clinical studies of good quality, one of which demonstrated dose-dependent efficacy (Chrubasik et al., 2000). A recent study was not able to confirm clinical effectiveness in the treatment of osteoarthritic and rheumatoid arthritic pain (Biegert et al., 2004)—for various reasons. The more intensive the pain, a higher dose is necessary. Our study (author SC) showed that the extract Assalix^R with 720 mg salicin per day was sufficient to relieve pain in patients with disk prolapses. However, safety data are necessary before such high doses can be recommended for the treatment of skeletal pain.

Willow bark extract does not damage the stomach mucosa or influence thrombocyte aggregation. Thus, it is not an acetylsalicylic acid substitute for cardioprotection. Specific adverse events other than occasional allergies have not yet been observed.

c) Preparations of Uncaria tormentosa (AUBLET) J. GMEL.

One exploratory study shows, that a standardized preparation containing 0.75 mg of pentacyclic oxindole alkaloids (Figure 9), Krallendorn^R (uncariae rhizoma) was effective in patients suffering from inflammatory rheumatic complaints (Mur et al., 2002). More studies are urgently required to prove clinical effectiveness and the tolerability of the extract in the treatment of osteoarthritic and low back pain.

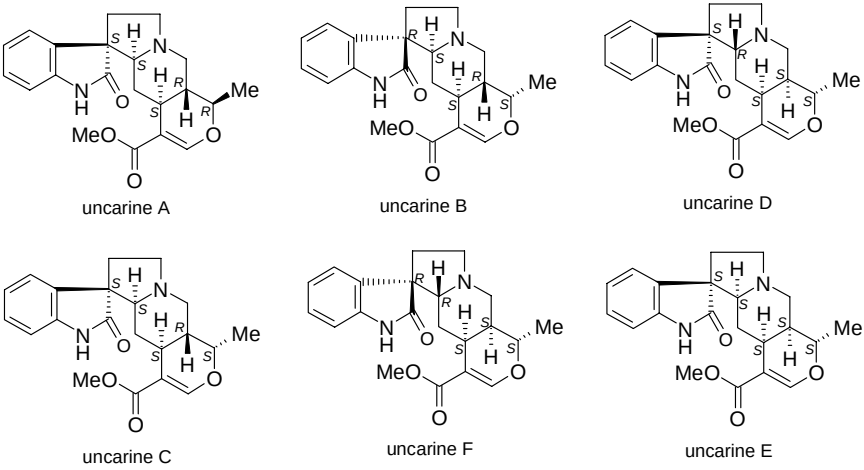


Fig. 9. The structure of pentacyclic oxindole alkaloids (co-active compounds) of *Uncaria tormentosa.*

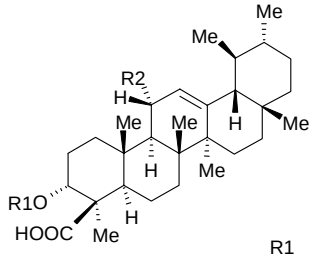
d) Avocado soybean fraction Piascledine^R

In France, intake of the unsaponifiable fraction of avocado (*Persea americana* MILL.) and soybean (Glycine max WILD.) is very popular amongst osteoarthritic sufferers. The fruit of the avocado consists of a large number of lipophilic compounds and is a rich source of monounsaturated food lipids and essential fatty acids (linoleic acid and α-linolenic acid). The active principles of avocado are linoleic acid (Figure 4) and α-linolenic acid (Figure 5) and other constituents in the unsaponifiable fraction. COX-2 inhibition and inhibition of hyaluronidase were demonstrated *in vitro* as well as interaction with cytokine release (Table 1a and 1b). Several

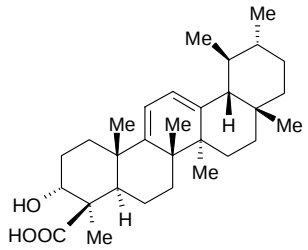
clinical studies of good quality have been summarized in a systematic review (Ernst, 2003) and showed good evidence for the effectiveness of the unsaponifiable fraction of avocado and soybean in the treatment of osteoarthritic complaints. Although a cartilage-preventative effect was found in experimental osteoarthritis, a long-term study over two years was not able to prove the cartilage-damage-preventative effect of the fraction except for a subgroup with less severe complaints (Lequesne et al., 2002). This needs further evaluation. To-date, specific adverse events have not been reported.

Use of herbal medicine in the treatment schedule

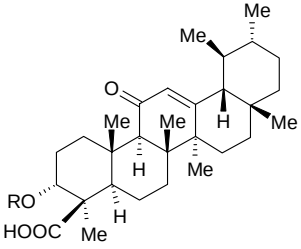
According to the guidelines of the American College of Rheumatology (www.rheumatology.org/publications/guidelines/oa-mgmt/oa-mgmt.asp?aud=mem) all possibilities with less adverse events than synthetic medications should be tried as the first line treatment of pain and inflammation. Use of the plant materials as self-medication, herbal food supplements and herbal medicinal products should, thus, be



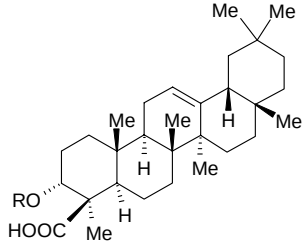
	R1	R2
β-Boswellic acid	H	H
3-*O*-Acetyl-β-boswellic acid	Ac	H
3-*O*-Acetyl-11-α-methoxy-β-boswellic acid	Ac	OMe



9,11-Dehydro-β-boswellic acid



	R
11-Keto-*β*-boswellic acid	H
3-*O*-Acetyl-11-keto-β-boswellic acid	Ac



	R
α-Boswellic acid	H
3-*O*-Acetyl-α-boswellic acid	Ac

Fig. 10. The structures of boswellic acids—co-active compounds of the active principle in *Salai guggal*.

recommended before synthetic medications are advised. It is suggested that if no improvement occurs within three to four months intake of a herbal medication, other herbal preparations or a combination of herbal medicines should be tried. So far the evidence of effectiveness of herbal medicinal products is strong for preparations from *Harpagophytum procumbens* and avocado soybean, moderate for ginger and rose hip and, conflicting for willow bark. For preparations from *Salai guggal (Boswellia serrata* ROXB. ex COLEBR.), no evidence of effectiveness is available, although theoretically it may alleviate osteoarthritic complaints (Table 2; Chrubasik et al., 2007a). Boswellic acids (Figure 10) have been shown to specifically inhibit 5-lipoxygenase and were identified as a part of the active principle. Future research may provide an improved extract from this herbal medication to improve osteoarthritic conditions.

Table 2. Number of systematic reviews and their conclusions on the evidence of effectiveness of medicinal product from various herbs (after Chrubasik et al., 2007a)

	No.	*Evidence*
Devil's claw	5	Strong
Avocado soybean	1	Strong
Ginger	1	Moderate
Rose hip and seed	1	Moderate
Willow bark		Moderate
Salai guggal	1	Insufficient
for other preparations	6	Insufficient

Future perspectives

Herbal extracts have been shown to be more potent in alleviating pain and inflammation than individually isolated compounds. In addition they are associated with less adverse events than isolated/synthetic compounds probably due to the lower dose in the active principle and the broader mechanism of action of the co-active compounds. Future research should focus on the preparation of improved extracts that concentrate the active principle and free them of inactive compounds especially compounds that are less well tolerated or those that cause adverse events. Plant cultures may provide starting materials with higher content of co-active compounds. Aspects of good manufacturing practice and good laboratory practice are a matter of course.

Randomized controlled studies according to good clinical practice needs to be carried out to increase the body of evidence for the effectiveness

of herbal medicines in the treatment of pain and inflammation. Surveillance and pilot studies should be followed by confirmatory studies that prove the efficacy of the herbal medicinal products. A comparison of herbal medicinal products in homogenous patient populations may enable a ranking of the products in different osteoarthritic conditions.

Currently, there are not sufficient safety data available for any of the herbal medicines for pain and inflammation. Preclinical studies are urgently needed to rule out any harm, especially for chronic condition which requires treatment over longer periods. Likewise, data are scarce and very much needed to assure safety during pregnancy and lactation.

Conclusion

Treatment with herbal medicinal products is another treatment option with less adverse events compared to synthetic medications and should be considered as first-line treatment in the sense of the American College of Rheumatology that treatments with lower risks should be preferred. In case of musculoskeletal pain, three herbal anti-inflammatory options are available. The first option includes an inexpensive self-treatment with nettle herb (herba urticae), blackcurrant leaf (ribes nigri folium) or raw ginger (zingiberis rhizoma). For pain relief, nettle leaf may be rubbed onto the affected area or a stew of nettle herb may be consumed. A tea from blackcurrant leaf or freshly squeezed ginger may also help to save on synthetic analgesic medications.

The second option are food supplements with moderate evidence of effectiveness such as the rose hip powder Litozin^{R} and the γ-linolenic acid containing seed oils from borage (*Borago officinalis* L.), evening primrose (*Oenothera biennis* L.) or blackcurrant (*Ribes nigrum* L.). Confirmatory studies that prove the effectiveness in the treatment of painful osteoarthritis have not yet been carried out.

The third option comprises the anti-inflammatory herbal medicinal products with good clinical evidence of effectiveness including Doloteffin^{R}, an aqueous extract from devil's claw (*Harpagophytum procumbens* **(**Burch.) DC. and the unsaponifiable fraction Piascledine^{R} from avocado and soybean. For other anti-inflammatory herbal medicinal products, the evidence is conflicting: for the ethanolic extract Assalix^{R} from willow bark (*Salix purpurea* L. *subspecies daphnoides*) or insufficient: for the extract Krallendorn^{R} from cats's claw (*Uncaria guianensis* (AUBLET) J. GMEL.) or for preparations from *Salai guggal* (*Boswellia serrata*).

Confirmatory studies to increase the body of evidence of effectiveness as well as safety data and research on the mechanism of action are urgently needed for increased treatment acceptance of herbal medications.

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13 Metabolic Engineering for the Fabrications of Pharmaceutically Central Metabolites from Microorganisms and Higher Plants

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Introduction

For millions of years nature has involved in chemical evolution through a natural combinatorial synthesis. Plants are very interesting and useful sources of molecules for the discovery of new medicinal products.

One of the most important fundamental objectives of metabolic engineering or metabolomics is the augmentation of cellular phenotype, for instance metabolite over-production, by the introduction of genetic controls.

The era of recombinant approaches to metabolic engineering of natural products has a very definable beginning.

The completion of sequencing of the several genomes including the human genome has resulted in the increased number of availability of genes for metabolic engineering. In parallel, increased number of databases and *in silico* (bioinformatic) tools to handle these genomes even in a large scale, also help to develop more precisely the metabolomics.

In this chapter some general and technical aspects of metabolomics are reviwed in the framework of designing novel leads or producing large amounts of pharmaceutically as well as other industrially important compounds, which are complicated to manufacture using usual synthetic approaches and less amount present in nature. Recent advancements in the field of metabolomics are also reviewed.

Metabolic engineering has proven to be a precious instrument for the large production of several pharmaceutically as well as industrially relevant molecules from microorganisms as well from higher plants. Current development of studies employing transgenic and/or recombinant technologies have opened up opportunities of the metabolic engineering of biosynthetic pathways, leading to the growth of manufacturing high-value secondary metabolites, even at the commercial level.

By definition 'metabolomics' is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind"—specifically, the study of their small-molecule metabolite profiles (Daviss, 2005). The word 'metabolome' corresponds to the set of all metabolites in a particular living organism; which are the final products (e.g., secondary metabolites, metabolic intermediates, hormones, proteins, or any signaling molecules, etc.) of its' gene expression. Although the messenger RNA (mRNA) gene expression data and proteomic analyses do not tell the complete chronicle of what possibly may be occurring in a cell, metabolic profiling is able to offer an immediate 'snapshot' of the physiology of that particular cell. One of the most important challenges of systems biology is to assimilate 'proteomics', 'transcriptomics', and 'metabolomics' information to present a further inclusive representation of the whole living organisms. The word metabonomics' is also used, particularly in the context of drug toxicity assessment. There is some disagreement over the exact differences between 'metabolomics' and 'metabonomics' ; in general, the term 'metabolomics' is more commonly used (Anonymous, 2005).

The chemical evolution of nature can be regarded as a natural combinatorial synthesis for millions of years. During this period, higher plants evolved natural products with remarkable pharmacological activities. Compounds obtained from natural sources such as micro-organisms and plants make up as much as 40% of the drugs in current

use, either directly or through synthetic modification, and have a prominent place in the history of drug discovery (Hutchinson, 1998).

Plants manufacture a huge array of pharmaceutically significant molecules. Many of these are derived from plants which are complicated to cultivate, or grow exceptionally slowly. In the past there has been a comprehensive study for the potential to manufacture these kinds of molecules by using different techniques of metabolomics, like cell culture, etc.

During the molecular evolution, plants have developed very efficient approaches to magnetize pollination or seed-dispersing animals (such as, birds or insects), for shielding themselves against UV-light and for defending themselves against herbivores, microorganisms and other plants. In natural growth conditions, plants compete for space, light, water and nutrients. The major strategies used are based on the production of secondary metabolites, including, essential oils, which are fragrant products active either as attractant for pollinations or as a repellent. The pollination is also favoured by colourants like molecules such as, flavonoids, anthocyanins and carotenoids. Flavonoids are also involved in the absorption of UV-radiation (Meijkamp et al., 1999) and in many defensive processes.

The term 'metabolic engineering' coined by J.E. Bailey (1991), exemplifies a broad array of manipulations and experimental advances to improve the productivity of a desired metabolite by an organism (Strohl, 2001). Included under the banner of metabolic engineering are examples of improvement in productivity and/or yield, improvement of substrate uptake, broadening the substrate range of an organism, improvement of the suitability of an organism in fermentations (i.e., 'fitness'), analysis and

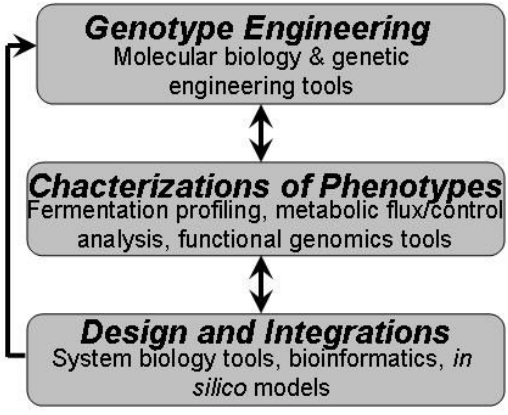

Fig. 1. Different tools and steps involved in the metabolic and reverse metabolic engineering.

modification of metabolic flux, elimination of metabolic overflow, elimination of unwanted or competing metabolic pathways, improvement of the organism to resist noxious conditions such as solvents, and so forth (Stephanopoulos, 1999).

The very different varieties of biochemical pathways and production of secondary metabolites is the basis for the well-known feature of plants, which are a tremendous source for isolation of molecules and of great interest for the discovery of new medicinal agents, considered less risky than those of microbial or animal origin (Shanks and Morgan, 1999). However, it should be pointed out that the beneficial medicinal effects of plant material might be also due to the endogenous combination of several secondary products. On the other hand, approaches involved in genetic engineering of medicinal plants have been ascribed to increase yield of pharma-ceutically important single secondary metabolites (Briskin, 2000).

Table 1 presents some plant-derived pharmaceutical products, including the source plant species, their price in US$ and their therapeutic indications.

The plant molecular biology has evolved since long time ago from studies of single gene functions useful to extrapolate basic principles in a very simplistic manner, to more analysis of processes known to be regulated by a multitude of tightly interconnected and even interdependent factors. By definition, the modern plant molecular science, and in particular its applied component plant biotechnology requires a holistic, multi-disciplinary and comprehensive approach to achieve its objective (Sasaki and Christou, 2004).

The enhanced classification of the biomolecular complexes, branching, and the knowledge of the interactive metabolic pathways of many organisms is feasible with advances in genome sequencing, transcriptome and proteome analysis and may provide more suitable starting platforms for engineering of secondary metabolite production. Indeed, it is knowledge and exploitation of the dynamic nature of gene expression and enzyme regulation that may prove to be highly useful in metabolic and combinatorial engineering approaches. In combination with additional techniques for metabolic pathway modification, such as directed evolution, the goals of improved secondary metabolite production is the employment of novel products which can be readily achieved (Mijts and Schmidt-Dannert, 2003).

Over the past six years, several review papers have been published on the subject of metabolic engineering of natural products (Strohl, 2001).

In this chapter some of the recent advancements in this field and the general and technical aspects of metabolomics (metabolic engineering) are presented in the context of designing novel lead molecules or to produce

Table 1. Pharmaceutical products from natural sources (mainly from plants) (modified from Rao and Ravishankar 2002)

Plants	*Compounds*	*Therapeutic uses*	*Price (US$/kg)*
Aconitum spp.	Acinitine	Antiarhythmic	n/a[a]
Artemisia annua	Artemisinin	Antimalarial	400
Coptis japonica	Berberine	Intestinal ailment	3,250
Capsicum frutescens	Capsaicin	Counter-irritant	750
Camptotheca acuminata	Camptothecin	Antitumor	432,000
Castanospermum australe	Castanospermine	Glycoside inhibitor	n/a[a]
Catharenthus roseus	Vincristine	Antileukemic	2,000,000
Catharenthus roseus	Vinblastine	Antileukemic	1,000,000
Catharenthus roseus	Ajmalicine	Antihypertensive	37,000
Cinchona ledgeriana	Quinine	Antimalarial	500
Colchium autumnale	Colchicine	Antitumor	35,000
Coleus forskolii	Forskolin	Bronchial asthma	n/a[a]
Digitalis lanata	Digoxin	Cardiac stimulant	3,000
Dioscorea deltoidea	Diosgenin	Steroidal precursor	1,000
Lithospermum erythrorhizon	Shikonin	Antibacterial	4,500
Orchrosia elliptica	Ellipticine	Antitumor	240,000
Papavar somniferum	Codeine	Sedative	17,000
Papavar somniferum	Morphine	Sedative	340,000
Panax ginseng	Ginsenosides	Health tonic	n/a[a]
Podophyllum petalum	Podophyllotoxin	Antitumor	n/a[a]
Sanguinaria canadensis	Sanguinarine	Antiplaque	4,800
Taxus brevifolia	Taxol	Anticancer	600,000

Notes: [a]not available.

huge amounts of pharmaceuticals as well as other industrially important compounds which are difficult to develop even with usual synthetic approaches.

Reasons for considering biotechnology in metabolic engineering

The biotechnological approaches generally consist of growth of cells, tissues, organs or the entire organisms by growing them *in vitro* and genetic manipulation of them to get desired compounds. The development of the micropropagation methods for a number of medicinal plant species has been already reported and needs to be extended (Naik, 1998).

Cryopreservation of cells is an area of great interest in the preservation of medicinal plants and it has already been used in several plant species. The development and adoption of plant cell culture and organ culture methods have led to the production of plant products on a large scale (Rao and Ravishankar, 2002).

The fundamental ambition of metabolic engineering is the enhancement of cellular phenotype, such as metabolite over-production by the introduction of genetic controls. At this point, metabolic engineering endeavours have considered the properties of the overall metabolic network, in sharp distinction to the single-gene focus that characterizes emblematic applications of genetic engineering, as well as the biotechnology (Alper et al., 2005).

Table 2 shows different issues related to the biotechnological advances in the usual projects found on plant-derived secondary metabolites. Table 3 presents some of the classes and sub-classes of compounds isolated and reported so far from the higher plant cultures *in vitro*.

Table 2. Partial list of the most important issues of biotechnology in the production of secondary metabolites from plants (from Rao and Ravishankar, 2002)

Main issues	*Related issues*
Plant cells, tissues and organ cultures	Cell culture Shoot culture Root culture Scale-up of cultures
Transgenic plants/organisms	Metabolic engineering Heterologous expression Generation of molecular complexes
Micro-propagation of medicinal plants	Endangered plants High-yielding varieties Metabolically engineered plants
Newer sources	Algae Other photosynthetic marine forms
Safety considerations	Propagation in the cell environment Effects on biodiversityEffects on health

Table 3. Examples of the chemical classes and sub-classes of compounds isolated from higher plant cultures *in vitro* (modified from Stockigt et al., 1995)

Chemical classes of compounds				
Phenylpropanoids	*Alkaloids*	*Terpenoids*	*Quinones*	*Steroids*
Anthocyanins	Acridines	Monoterpenes	Anthro-quinones	Cardiac glycosides
Coumarins	Betalaines	Sesquiterpenes	Benzo-quinones	Pregneno-lone derivatives
Flavonoids	Quinolizidines	Diterpenes	Naphtho-quinones	
Hydroxy-cinnamoyl derivatives	Furono-quinones	Triterpenes		
Isoflavonoids	Harringtonines	Carotenes		
Lignans	Isoquinolines			
Phenolenones	Indoles			
Proanthocyanidins	Purines			
Stilbenes	Pyridines			
Tanins	Tropane alkaloids			

Major issues and new trends in metabolic engineering

Genetic modification by plant transformation allows stable alterations of the biochemical processes involved in important traits such as yield, nutritional quality and resistance to diseases. The achievements in modification of secondary metabolism in plants have been reviewed, including alternative approaches to change the secondary metabolism (Hallard, 2000).

There are at least three distinct uses of metabolic engineering (metabolomics) in plants:

1. Increase or decrease of the amount of metabolites and production of 'novel' compounds;
2. Improving quality traits of food and fodder or alter traits of horticultural plants;
3. Increasing the resistance of plants against pest and diseases.

1. In the first strategy of metabolomics the aim is to increase the level of certain compounds, like medicinal products, colourants, flavours and

fragrances, for economically important production. For example, strictosidine synthase (*Str*) has been over-expressed in *Catharanthus roseus* leading to the production of 200 mg/ml terpenoid indole alkaloids (Canel et al., 1998). Another interesting example is that of the stable transgenic hairy root clone of *Hyoscymus muticus*, over-expressing the hyscyamine 6β-hydroxylase (H6βH); this transformation ultimately leads to the production of 17 mg/ml scopolamine, which was about 100 times more than the wild-type clones (Jouhikainen et al., 1999). In another example, a new pathway was created by introducing a gene encoding a biosynthetic enzyme like *Tdc* (tryptophan decarboxylase) from *Catheranthus roseus*, in tobacco resulting in a high production of tryptamine (up to 1 mg/gm fresh weight) (Songstad et al., 1990).

2. The second approach consists of development of dietary values of the foodstuff by activating pathways leading to health endorsing molecules. For instance, following genetic engineering approaches the pro-vitamin A (β-carotene) biosynthetic pathway has been activated into rice endosperm, in order to facilitate the production of vitamin A in the rice, reducing a serious health problem due to vitamin A deficiency in many countries using rice as the chief staple-diet (Hallard, 2000).

3. The third issue is the use of metabolomics of secondary metabolism to increase or broaden the spectrum of disease resistance. An example is the introduction of the biosynthesis of novel phytoalexins or structural variants of the naturally occurring phytoalexins, or by modifying expression of transcriptional regulators of phytoalexin pathways. For instances, the introduction of stilbene synthase gene in tobacco and tomato has increased the resistance against *Botrytis* and *Phytophthora* infection, respectively. This protection was due to the production of the phytoalexin-resveratrol (Hallard, 2000).

Genetically modified medicinal plants

Metabolic engineering of natural products is a science that has been built on the goals of traditional strain improvement with the availability of modern molecular biological technologies. In the past 15 years, the state of the art in metabolic engineering of natural products has advanced from the first proof-of-principle experiment based on minimal known genetics to a commonplace event using highly specific and sophisticated gene manipulation methods. With the availability of genes, host organisms, vector systems, and standard molecular biological tools, it is expected that metabolic engineering will be translated into industrial reality (Strohl, 2001).

The genetic transformation allowing plants technologies are versatile

platform for cultivar improvement as well as for studying gene function (Hansen and Wright, 1999).

Transgene integration in plants transformed by either *Agrobacterium*-mediated or direct DNA delivery methods occurs via illegitimate recombination (Sasaki and Christou, 2004). Recent investigations aimed at genetically dissecting transgene integration mechanism(s) have provided new insights into the process and are expected to fast track the elucidation of factors that control stable and predictable transgene expression (Sasaki and Christou, 2004).

It is surprising that relatively few metabolic pathways have been modulated using recombinant DNA technology. One reason for this is that such pathways must be studied in the context of the whole cell, rather than at the single pathway level, and that even the simplest modifications can have unpredictable consequences throughout the complete system (Sasaki and Christou, 2004).

Genetic engineering of a secondary metabolic pathway aims to either increase or decrease the quantity of a single compound or group of interconnected compounds. To decrease the production of a certain unwanted (or of a group of) compound(s) several approaches have been reported. An enzymatic step in the pathway can be knocked out, for example, by reducing the level of the corresponding mRNA via antisense, or RNA interference (RNAi) technologies, or by over-expressing an antibody against the enzyme (Verpoorte and Memelink, 2002). The antisense approach has been successfully used for changing flower colours (Verpoorte and Memelink, 2002). In this case other approaches include diversion of the flux into a competitive pathway or an increase in the catabolism of the target compound (Verpoorte and Memelink, 2002).

NPTII is one of the first widely used dominant selectable markers in eukaryotes. It encodes neomycin phosphotransferase (NPT) conferring resistance to amynoglycosides (for structure see Figure 2). This family of

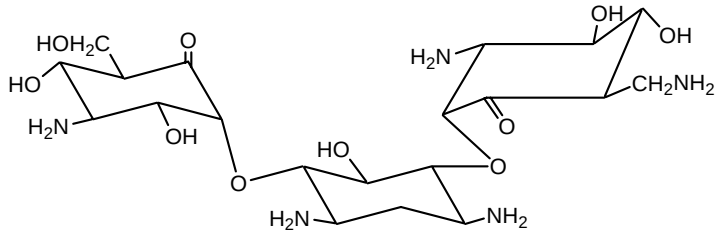

Fig. 2. The amynoglycoside structure of the antibiotic kanamycin B.

antibiotics, which includes kanamycin, neomycin and gentamycin, interact in the cytosol of prokaryotes and eukaryotes with at least three ribosomal proteins and with specific bases within the decoding region of the smaller ribosomal RNA subunit, resulting in inhibition of protein synthesis and increased frequency of induced translational errors. These activities determine in fact the observed *toxicity* of the selective media upon the viability of the plant tissues (Cucu et al., 2002).

Cucu and co-workers (2002) performed an indirect and mediated genetic transformation of *Atropa belladona* using the disarmed *Agrobacterium tumefaciens* pTi carrying the *NPTII* gene for neomycin phosphotransferase. Its expression into the plant host genome conferred the kanamycin resistance trait which has been easily detected by both *in vitro* and molecular-genotype (PCR) and phenotype (protein) assays. The obtained genotype and phenotype profiles may confirm both the integration and the expression of the *NPTII* gene into the *A. belladonna* genome (Cucu et al., 2002).

More often, the goal is to increase the production of certain compounds in the normal producing plant species (Verpoorte and Memelink, 2002). The overproduction or enhancement of desired metabolites, however, is largely a different issue than the production of novel metabolites. Overproduction can be addressed by increasing the critical precursor pool(s), by either adding, modifying, or deleting regulatory genes, by altering promoter, terminator, and/or regulatory sequences, increasing the copy number of genes for (a) bottleneck reaction(s), or removing competing, unneeded pathways (Strohl, 2001).

A good example of pathway-specific metabolic engineering comes from the detailed metabolic flux analysis of precursors required for cephamycin and cephalosporin biosynthesis in *Streptomyces clavuligerus* by Malmberg et al. (1993 and 1995). Finding that α-aminoadipic acid (α-AAA) was a limiting precursor, they cloned an extra copy of the lysine ε-amino-transferase (LAT) gene into the chromosome of *S. clavuligerus*, achieving both increased enzyme activity and significantly greater levels of the desired product cephamycin C (Malmberg et al., 1993; Malmberg et al., 1995; Khetan et al., 1996).

Over the past five years, the field of metabolic engineering of natural products has seen an exponential proliferation of efforts from an ever-expanding cadre of laboratories. Additionally, industrial efforts have also been rejuvenated, largely due to the interest of small biotechnology companies and the maturation of the technology used to develop such compounds (Strohl, 2001).

Technologies for genetic transformation of plants

Genetic transformation of plants are possible using different DNA delivery technologies, the most successful of which (Hallard, 2000) are (a) the introduction of genes via a binary vector transferred by *Agrobacterium*, and (b) direct delivery strategies.

Agrobacterium is a plant pathogenic bacterium occurring in the soil that has the ability to transfer part of its DNA into the genome of the plant. Two species of *Agrobacterium* are of particular importance in plant tissue culture, *A. tumefaciens* (which causes the formation of an undifferentiated tumor, known as 'crown gall tumor') and *A. rhizogenes* (which induces the formation of fast-growing plagiotropic, highly branched root tissue, known as 'hairy root'). Both of these phenotypes are the results of the ability of the plasmid of *Agrobacterium* to incorporate several of its genes into the genome of host plant (Hallard, 2000).

As far as founded gene transfer approaches for direct uptake of DNA by protoplasts, several methods were developed and reported (Kofer et al., 1998; Hallard, 2000). Since DNA is not lipophilic, it is relatively incapable of passing through the plasma membrane without some treatment, to increase permeability such as with polyethylene glycol (PEG). The PEG method for transformation allows the insertion of the DNA in the plastome. In case of plasmid transformation, this can be accomplished by biolistic and PEG transformation technologies (Kofer et al., 1998; Hallard, 2000).

Furthermore, DNA uptake into protoplasts can also be induced electrically (electroporation) in order to produce temporary pores in the plasma membrane (Hallard, 2000).

Some other alternative DNA delivery methods have been reviewed by Songstad et al. (1995). They reviewed recent updates of silicon carbide fibers, electroporation, electrophoresis, microinjection, etc., and associated advantages and disadvantages (Hallard, 2000).

Genomic tools

Up to April 1999, the sequences for more than 150 different gene clusters encoding natural product biosynthesis pathways had been placed into the public data bases (at The National Center for Biotechnology Information, NCBI) (Strohl, 2001). At that point, the prokaryotes were represented by 115 gene clusters, of which approximately half encoded Type I or Type II polyketide biosynthesis pathways, and another 400 encoded non-ribosomal peptide biosynthesis pathways (NRPS mediated) (see Table 4) (Strohl, 2001).

With the recent advances in sequencing brought about with automated

sequences such as, the ABI Prism 3100 Genetic Analyzer, which is capable of handling a through-put of 184,320 bases per day, genomes are being sequenced today at nearly the rate that natural product gene clusters were being sequenced just 5 years ago. Thus, the database and number of genes in it is expected to grow exponentially over the next few years. This wealth of information, as well as the sequences themselves, will allow more accurate and informative bioinformatic analyses, as well as a much larger pool of genes that can be used to manipulate natural product biosynthetic pathways (Strohl, 2001).

Table 4. Analysis of gene clusters encoding the biosynthesis of natural products in microorganisms (from Strohl, 2001)

DNA source encoding	*Number of gene clusters*
Prokaryotic pathways	
Total	115
Type I polyketides	22
Type II polyketides	25
Non-ribosomal peptide synthetases (NRPS)	40
Aminoglycosides	11
Other	17
Eukaryotic pathways	
Total	41
Type I iterative polyketide synthases (IPS-I)	15
Non-ribosomal peptide synthetases (NRPS)	13
Isoprenoid	12
Other	1

In a review Cameron and Chaplen in 1997 stated that, the complete sequencing of several microbial genomes has resulted in the increased availability of genes for metabolic engineering. The number of databases and computational tools to deal with this information has also increased. This development has stimulated, and will continue to stimulate, advances in metabolic engineering. Specific recent advances include improvement of pathways for aromatic metabolites, the development of a more complete understanding of the effect of bacterial hemoglobin on cell performance, the development of NMR-based methods for the monitoring of intracellular metabolites and metabolic flux, and the application of metabolic control analysis and metabolic flux analysis to a variety of systems (Cameron and Chaplen, 1997).

Combinatoral biosynthesis for new drug discovery

Combinatorial biosynthesis involves interchanging genes responsible for secondary metabolism between antibiotic-producing microorganisms to create unnatural gene combinations or hybrid genes if only part of a gene is exchanged (Hutchinson, 1998).

It is possible to make novel metabolites by utilizing both the approaches, due to the effect of a new enzyme on a metabolic pathway or to the formation of proteins with new enzymatic properties. The method has been particularly successful with the polyketide synthase (PKS) genes: derivatives of medically important macrolide antibiotics and unusual polycyclic aromatic compounds have been produced by novel combinations of the type I and type II PKS genes, respectively. Recent extensions of the approach to include *deoxy*-sugar (DOS) biosynthesis genes have expanded the possibilities for making new microbial metabolites and discovering valuable drugs through the genetic engineering of bacteria (Hutchinson, 1998).

The PKS genes, which direct the development of an especially large family of secondary metabolites (O'Hagan, 1991), were the first genes used to clone homologous genes representing a specific class of microbial natural products (Malpartida et al., 1987). The DOS biosynthesis genes also have been employed in the same manner (Stockmann and Piepersberg, 1992); in fact, many other types of secondary metabolism genes can be used in a similar fashion as a fast way to access the gene cluster (Hutchinson, 1998). The availability of the first cluster of antibiotic production genes cloned triggered research to discover whether the genes could be expressed in another microorganism. This was successful (Malpartida and Hopwood, 1984) and led to the important investigation of whether genetically hybrid bacteria containing one or more genes from two different organisms that allow biosynthetically related metabolites to produce novel ones (Hutchinson, 1998).

Metabolic engineering of lycopene biosynthesis: a case study

Lycopene (for chemical structure see Figure 3), a carotenoid in the same family as β-caroteno, is what gives tomatoes pink, grapefruit, apricots, red oranges, watermelon, rosehips, and guava their red color. It is not merely a pigment. It is a powerful antioxidant that has been shown to neutralize free radicals, especially those derived from oxygen, thereby conferring protection against prostate cancer, breast cancer, atherosclerosis, and associated coronary artery disease. It reduces LDL (low-density lipoprotein) oxidation and helps reduce cholesterol levels in the blood (Helmenstine, 2005).

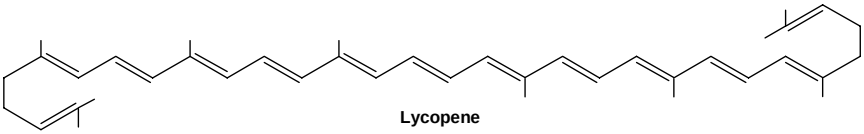

Fig. 3. The structural features of lycopene.

Very recently Alper and co-workers (2005) identified the gene responsible for producing lycopene in *Escherichia coli*. The authors constructed a triple knockout gene which can ultimately exhibited a nearly 40% increase over an engineered, high producing parental strain (Alper et al., 2005).

For this purpose Alper et al. (2005) utilized genome-wide stoichiometric flux balance analysis as an aid in discovering putative genes impacting network properties and cellular phenotype. In the case of lycopene biosynthesis in *E. coli*, they constructed and tested corresponding single, double and triple gene knockouts.

Metabolic engineering in industry

The large-scale production of antibiotics, anticancer agents, and other important drugs from microbial fermentations has been a cornerstone of the pharmaceutical industry since the development of penicillin in the 1940s. Since natural microbes do not produce compounds of pharmaceutical interest in large scale for commercial purposes, strain improvement became a critical part of drug development process (Vinci and Byng, 1999; Parekh et al., 2000; Baltz, 2001). Metabolic engineering technology for industrial microorganisms is under development to create rational, more reliable, and more cost-effective approaches to strain improvement. Strain improvement is a critical component of the drug development process, yet the genetic basis for high production by industrial microorganisms is still a mystery (Reeves et al., 2004).

Interestingly, the traditional mutate-and-screen method of strain improvement that was developed in the 1940s for the penicillin strain has not changed significantly over the years (Queener and Lively, 1986; van Nistelrooij et al., 1998). This is because the method is technically simple to perform and has been successful at generating improved strains. Today's penicillin strain, for example, has been improved 3000-fold over the strain used in the early 1940s (van Nistelrooij et al., 1998).

Reeves et al. (2004) began searching of genetic modifications critical for high-level antibiotic production. The model system was used for

erythromycin production, in the unicellular actinomycete, *Aeromicrobium erythreum*. A tagged-mutagenesis approach allowed reverse engineering of improved strains, revealing two genes, mutB and cobA, in the primary metabolic branch for methylmalonyl-CoA utilization. Knockouts in these genes created a permanent metabolic switch in the flow of methylmalonyl-CoA, from the primary branch into a secondary metabolic branch, driving erythromycin overproduction. The model provides insights into the regulation and evolution of secondary metabolism (Reeves et al., 2004).

'Inverse/Reverse' metabolic engineering

In 1996 Bailey and co-workers defined '*inverse metabolic engineering*' as, a particular host organism which is engineered to obtain improved properties by transferring the desired phenotype from another organism in which it is functional (Baily et al., 1996). Even when the transferred phenotype is not expressed in the host organism identically as in the original, the observed 'complications' can assist in obtaining information about the host that might lead to better identifying targets for genetic and metabolic modification (Lai and Klapa, 2004).

For bacteria that produce pharmaceuticals, industrial classical strain improvement programs have successfully generated mutant strains with 10-100X higher product titers (Lal et al., 1996), and these strains can be used for reverse engineering studies (Lum et al., 2004). For instance, transcript levels in production cultures of wild-type and classically improved strains of the actinomycete bacteria *Saccharopolyspora erythraea* and *Streptomyces fradiae* were monitored using microarrays of the sequenced actinomycete *S. coelicolor*. *Sac. erythraea* and *S. fradiae* synthesize the polyketide antibiotics erythromycin and tylosin, respectively, and the classically improved strains contain unknown overproduction mutations (Lum et al., 2004).

Lum and co-workers stated that, the study of 'reverse engineering' addresses two key drawbacks of the traditional approach (Lum et al., 2004). These are:

(1) Traditional strain improvement programs are laborious and require long and unpredictable durations, typically many years;

(2) The random mutations in traditionally improved strains responsible for high titers cannot be identified readily, making overproduction mechanisms inaccessible for new strain engineering efforts.

By analyzing several thousands of genes in parallel, genomic technologies, especially the microarray-type technologies, should greatly facilitate the characterization of existing over-producer strains and generate

information to more efficiently engineer new strains that synthesize high yields of natural products (Lum et al., 2004).

Genome-wide or large-scale methodologies employed in functional genomics such as DNA sequencing, transcription profiling, proteomics, and metabolite profiling have become important tools in many metabolic engineering strategies. These techniques allow the identification of genetic differences and insight into their cellular effects. In the field of inverse metabolic engineering mapping of differences between strains with a different degree of a certain desired phenotype and subsequent identification of factors conferring that phenotype are essential parts. Therefore, the tools of functional genomics in particular have the potential to promote and expand inverse metabolic engineering. In a scientific report Bro and Nielsen (2004) stated the use of functional genomics methods in inverse metabolic engineering, and discussed the identification of targets for metabolic engineering with low fold changes using these techniques.

Conclusions and recommendations

In conclusion it can be stated that higher plants are still an important source of natural products with interesting and new pharmacological activities. In addition, other groups of organisms are worth investigating, such as lower animals or the marine floras.

Plant cell and transgenic hairy root cultures are promising potential alternative sources for the production of high-value secondary metabolites of commercial relevance. Recent developments in transgenic research have opened up the possibility of the metabolic engineering of biosynthetic pathways to produce high-value secondary metabolites (Rao and Ravishankar, 2002).

New genomic tools could be used to characterize intermediate strains in classical strain improvement programs, identifying strains that likely harbor different over-production mechanisms (Lal et al., 1996).

For improved and quicker strain development, scientists are now choosing 'reverse metabolic engineering' approaches. These approaches also address the major drawbacks of the classical 'metabolic engineering' techniques to save time, money, etc. So those still following the so called 'classical' approaches should consider the 'reverse' approaches.

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14 Pharmacogenomics of Biotic and Abiotic Natural Products Derived from Traditional Chinese Medicine for Cancer Therapy

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Introduction

Cancer is still the second-leading cause of death in industrialized countries. The fate of many cancer patients, for whom cure of their disease is not a reality, is becoming ever more of an issue. There are unprecedented efforts to discover new treatment, and the knowledge of cancer has increased dramatically over the past four decades. Natural products are a valuable resource for drug discovery and development.

The recent developments in drug discovery from natural resources can be traced back to fundamental observations in the first half of the 20th century. The term 'allelopathy' coined by the plant physiologist Hans Molisch in 1937 describes the effect of products of a donor plant on abiotic and biotic environmental factors. In line with this term is the name 'allelochemics' coined by Whittaker and Feeny in 1971 for compounds by which organisms of one species affect the growth, health, behavior or population biology of other species, excluding nutrients and vitamin-like compounds. Typically, allelochemicals are secondary metabolites, which are—in contrast to primary metabolites—not essential for the nutrition of plants. Secondary metabolites are regarded as defense against competitors, herbivores and pathogens and signal compounds to attract insects for reproduction. Therefore, secondary metabolites represent an important part

of the plants' life strategies to maintain survival and reproductive fitness. Fortunately, many secondary metabolites of terrestrial and marine organisms exert pharmacological features. It is, however, quite clear that the pharmacological activities of secondary metabolites does not represent an altruistic behavior of other organisms towards humans but rather a pleasing side effect.

Although medicinal herbs gradually lost importance in the course of chemistry's progress in industrialized countries during the 20th century, the current thriving revival of phytotherapy is followed by an increasing scientific interest in bioactive compounds as lead drugs for semi-synthetic modification.

It comes as no surprise that natural products belong to the major players in cancer research, since a considerable portion of anti-tumor agents currently used in the clinic are of natural origin. Drugs of different classes are part of the armory to fight the war against cancer, e.g., *Vinca* alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), camptothecin and its derivatives (topotecan, irinothecan), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin), and others (Zhang et al., 2000; Duflos et al., 2002; Hartmann and Lipp, 2006; Pommier, 2006; Cortés-Funes and Coronado, 2007; Levêque and Jehl, 2007). Over the past years, there was a major shift in the development of cancer drugs, from screening of cytotoxic drugs to the development of molecular targeted drugs. The conceptual idea is that the knowledge of the mechanism(s) of a drug provides a better approach to reach improved clinical results on the basis of patients' molecular characteristics.

These facts lead to pursue the search for new, bioactive lead structures with an anti-tumor activity by a strategy of bioactivity-guided isolation from plants used in traditional folk medicines. Medicinal plants are used in many tribes all over the world and are subjects of ethnobotany and ethnopharmacology (Heinrich and Bremner, 2006). It can be expected that the search for bioactive plant compounds is more successful in medicinal plants than a search across all plant species. Traditional Chinese medicine (TCM) commands a unique position amongst traditional medicines, since its enormous variety of drugs of plant origin is founded on more than 5000 years of documented tradition. An elaborated system has been developed and many written documents and textbooks have been handed down over the millennia. This might imply that inactive plants and recipes have vanished over the centuries and that the *materia medica* of modern TCM is enriched with bioactive plants. This may significantly improve prospects for identifying novel active constituents from TCM (Lee, 1999). Based on this rationale, we investigated natural products derived from TCM during

Fig. 1. TCM products are sold on traditional medicinal markets in China since many centuries. Today, they represent a valuable resource for the development of molecular targeted and individualized treatment options for cancer patients.

the past dozen of years (Efferth et al., 1996, 2001, 2002a, 2002b, 2003, 2004; Dell'Eva et al., 2004; Efferth, 2007; Efferth et al., 2007). Genome-wide microarray analyses provide an attractive approach to identify genes involved in the response of tumor cells to natural products. For example, we have investigated artemisinin and six derivatives thereof and found a significant correlation of the sensitivity of these compounds to genes regulating tumor angiogenesis (Anfosso et al., 2006). Indeed, one of the mechanisms of artemisinins towards tumors is the inhibition of angiogenesis as shown by us and others (Chen et al., 2003, 2004; Dell'Eva et al., 2004).

TCM comprises medicinal products from plants, animals and minerals, acupuncture, and other practices. In the present overview, we exemplarily focus on each one example from medicinal plants, animals, and minerals, in order to demonstrate the conceptual feasibility, how natural products derived from TCM can be investigated with cutting-edge technologies from molecular biology and pharmacogenomics.

Berberine from the Chinese goldthreat (*Coptis chinensis* Franch)

Berberine is the active principle of the Chinese Goldthreat (*Coptis chinensis*) and a number of other herbs. Berberine is used in traditional Chinese medicine for the treatment of dysentery and infectious diarrhea, and other diseases (Birdsall and Kelly, 1997). In addition to its anti-microbial activity (Amin et al., 1969; Ghosh et al., 1985; Sun et al., 1988; Lesnau et al., 1990; Nakamoto et al., 1990), a number of other pharmacological actions were reported including this term which is already included in antimicrobial, immuno-stimulant, anti-tumor, anti-motility, anti-parasitic, and hypotensive properties, and relaxant effects on the *Corpus cavernosum* in erectile dysfunction (Simeon et al., 1989; Lau et al., 2001; Drewes et al., 2003). Considering this broad spectrum of pharmacological activities, it is noteworthy that berberine is relatively nontoxic to humans (Rabbani et al., 1987).

In addition to the role of ABC transporters for berberine resistance of human tumor cells, they are also important for berberine's function in plants and microorganisms. For instance, berberine is synthesized in the roots of *Coptis japonica* and is transported by the ABC transporter, Cjmdr1, to the rhizome of the plant, where berberine protects from anti-microbial attacks (Shitan et al., 2003). Gram-negative plant pathogens have developed an effective permeability barrier against plant antimicrobials including berberine by a membrane which restricts the penetration of amphipathic compounds and by MDR pumps which extrude toxins across this barrier (Tegos et al., 2002). *Berberis* plants produce berberine as well as an ABC transporter inhibitor, 5-methoxy-hydnocarpin, to disable the bacterial resistance mechanisms against berberine (Stermitz et al., 2000).

In a recent investigation, we showed that berberine exerts profound cytotoxic activity towards tumor cell lines (Efferth et al., 2005a). The correlation of IC_{50} values for berberine of 60 NCI cell lines with those for daunorubicin, vincristine, paclitaxel, and other investigational drugs points to a possible involvement of berberine in the multidrug resistance phenotype. Given the relevance of ABC transporters for multidrug resistance (Gillet et al., 2004, 2007), a systematic analysis of the role of ABC transporter gene family for resistance to berberine has been undertaken. Significant relationships between the IC_{50} values for berberine to the expression of several ABC transporters in the 60 NCI cell lines indicate that they might be candidate resistance genes for berberine. Among different ABC transporters, the *ABCB1* (*MDR1*) gene raised our interest. We validated the results obtained by correlation analyses by using a cell model, which over-expresses this ABC transporter. Indeed, we found that *ABCB1* (*MDR1*) confers resistance to berberine, which is in accord to previous results of ours and others (Lin et al., 1999; Efferth et al., 2002b; Pan et al., 2002).

The response of tumor cells to berberine and protoberberine is determined by multiple other factors in addition to ABC transporters, i.e., activator protein 1 (AP-1), telomerase, DNA topoisomerase II, and nucleoplasmin/B23 (Fukada et al., 1999; Wu et al., 1999; Kang and Chung, 2002). For this reason, we performed COMPARE and hierarchical cluster analyses of microarray-based mRNA expression values for 9706 genes of the 60 cell lines of the National Cancer Institute (NCI), USA, in an effort to gain deeper insight into the multi-factorial nature of cellular response to berberine. Apart from genes with still an unknown function, we identified genes from different functional groups to be correlated with cellular response to berberine, *e.g.*, signal transducers (*MMP5, GRB14, FLJ13052*), interleukins (*IL-15*), and others (*GLS, CD2AP, ADCY8, NK4, SDHB, GC20*). Although none of these genes have been assigned to drug sensitivity, the results obtained from this study suggest that these genes may also contribute to berberine resistance. Further studies are warranted to clarify their causative relevance for cellular drug response. A recent study reported mRNA expression levels of 11,000 genes by microarray technology in relationship to cellular sensitivity to berberine (Iizuka et al. 2003). We compared our set of genes with that of these authors. None of the genes identified in the present study was among the genes identified by Iizuka et al. (2003). Two explanations might account for the discrepancy in both microarray-based investigations: first, while our approach focused on the correlation of mRNA expression levels in 60 NCI cell lines of different tumor types (leukemia, colon cancer, lung cancer, breast cancer, ovarian cancer, CNS tumors, prostate cancer, and renal cancer) and IC_{50} values for berberine, Iizuka et al. (2003) correlated gene expression of 8 pancreatic cell lines with response to berberine. The different tumor types used in both studies may lead to different gene expression profiles. Second, the selection criteria in our approach were very stringent. We selected only genes with the highest COMPARE indices and the smallest probability to correlate erroneously with IC_{50} values for berberine by false discovery rate (FDR) calculation. Other genes identified by Iizuka et al. (2003) might also correlate significantly with IC_{50} values for berberine in our approach, however with smaller COMPARE correlation coefficients. Furthermore, the evaluation of gene expression in the study of Iizuka et al. (2003) and in the present investigation was performed on a subset of 11,000 and 9706 genes, respectively, which represents only a fraction of an estimated total number of 25,000 genes in the human genome. As a result, there may be many genes not included that may play an important role in determining cell sensitivity to berberine.

Cantharidin from the Chinese blister beetle (*Mylabris phalerata* Pallas)

Cantharidin is the active principle of the Chinese blister beetle (*Mylabris phalerata*) which exerts profound cytotoxicity towards tumor cells. Topical application of cantharidin has a long tradition in Asian medicine for the treatment of warts caused by *Molluscum contagiosum* virus (MCV) infections (Moed et al., 2001), while the use of cantharidin to treat pediatric MCV infections in Western academic medicine has been found effective (Silverberg, 2003; Smolinski and Yan, 2005). Blister beetles and cantharidin are also used in China and Vietnam to treat cancer (Wang, 1989). Despite its usefulness, the potential poisonous effects of cantharidin would have fatal consequences in the event of careless mistakes in the use of this compound (Karras et al., 1996; Swingle et al., 2006). Not only is exact dosage required for the use of cantharidin itself, but also for raw preparations of blister beetles to be used in traditional medicines.

Cantharidin and norcantharidin (a demethylated cantharidin derivative, which also has clinical potential) are protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitors (Li and Casida, 1992; Honkanen, 1993; McCluskey et al., 2001; Rauh et al., 2007). This activity appears necessary for the growth inhibition activity against tumor cells of these compounds. Protein phosphatases are involved, among others, in the regulation of multiple cellular processes including apoptosis, signal transduction pathways, cell cycle progression, glucose metabolism and calcium transport (Wera and Hemmings, 1995). Thus, although the biochemical target of cantharidin and norcantharidin is known, the critical molecular pathways by which cantharidin and norcantharidin cause growth inhibition and cell death are unclear (Wu et al., 2001; Chen et al., 2005; Huan et al., 2006).

In a recent analysis, we showed that cantharidin treatment causes DNA strand breaks in CCRF-CEM cells (Efferth et al., 2005b). DNA strand breaks have also been documented in oral cancer KB cells after norcantharidin treatment (Kok et al., 2003). A correlation between an increase in the mRNA level for several DNA damage repair genes in the 60 cell line panel and resistance to cantharidin argues for DNA damage as a mechanistic component of cantharidin-induced apoptosis. This is consistent with a role for p53 in cantharidin-induced apoptosis seen in this study because one of the major functions of p53 is to induce apoptosis when DNA damage excesses a threshold (Norbury and Zhivotovsky, 2004); p53 also plays a role in norcantharidin-induced apoptosis in glioblastoma cells (Hong et al., 2000). Phosphorylation of p53 stabilizes the protein (Norbury and Zhivotovsky, 2004), thus inhibition of phosphatases that target p53 (see below) may enhance the ability of p53 to exert its effect. The ability of Bcl-2 to protect against cantharidin-induced apoptosis seen in this study

indicates that the DNA damage-triggered mitochondrial pathway is also involved. Mitochondrial dysfunction and activation of caspases involved in the intrinsic (mitochondrial) pathway of apoptosis have been seen in other cell types after cantharidin or norcantharidin treatments as well (Chen et al., 2002).

The cross-resistance of the oxidative stress resistant WEHI7.2 variants to cantharidin suggests that cantharidin treatment causes oxidative stress which plays a role in cantharidin-induced apoptosis. Analogs of cantharidin increase xanthine oxidase activity which would increase intracellular ROS (Tsauer et al., 1997). It is, therefore, tempting to speculate that oxidative stress is involved in the induction of DNA damage by cantharidin. Increase of endogenous ROS level has repeatedly been shown to cause significant DNA breakage (Slupphaug et al., 2003).

Protection against oxidative stress, an increased level of Bcl-2, or the presence of wild-type p53 have a modest affect on cantharidin-induced toxicity. Mutational inactivation of Polβ, but not of ERCC1, key enzymes of base excision repair and nucleotide excision repair pathways, respectively, exerted an effect on cantharidin cytotoxicity. This suggests that cantharidin induces non-bulky DNA lesions that are repaired by base excision repair, but not by nucleotide excision repair. Lesions induced by oxidative stress are rather repaired by base excision repair and DNA strand break repair pathways (involving homologous and non-homologous end-joining) (Christmann et al., 2003). This suggests the possibility that multiple mechanisms are responsible for cantharidin-induced toxicity. In this study, for example, p53 status affected the IC_{50} of cantharidin; however, cells with mutated p53 still died. A p53-independent mechanism of cantharidin-induced cytotoxicity has been seen in hepatoma cells (Chen et al., 2002).

In another investigation, we performed COMPARE analyses of the IC_{50} values for cantharidin and the microarray-based mRNA expression of 9706 genes in the 60 NCI cell lines to produce scale indices of correlation coefficients (Efferth, 2005). By this approach, 21 candidate genes were identified. The mRNA expression of these genes was subjected to hierarchical cluster analysis. The distribution of cell lines resistant or sensitive to cantharidin in the dendrogram was significantly different indicating that cellular response to cantharidin is predictable by these genes. Another conclusion from this study is that these candidate genes might contribute to the molecular action of cantharidin towards cancer cells. While the specific functions of the proteins encoded by the 21 genes identified by our approach are different, it is intriguing that many of them are in one way or another involved in DNA damage response, DNA repair, and/or apoptosis. Since cantharidin is an inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), the regulatory subunit 13B of PP1,

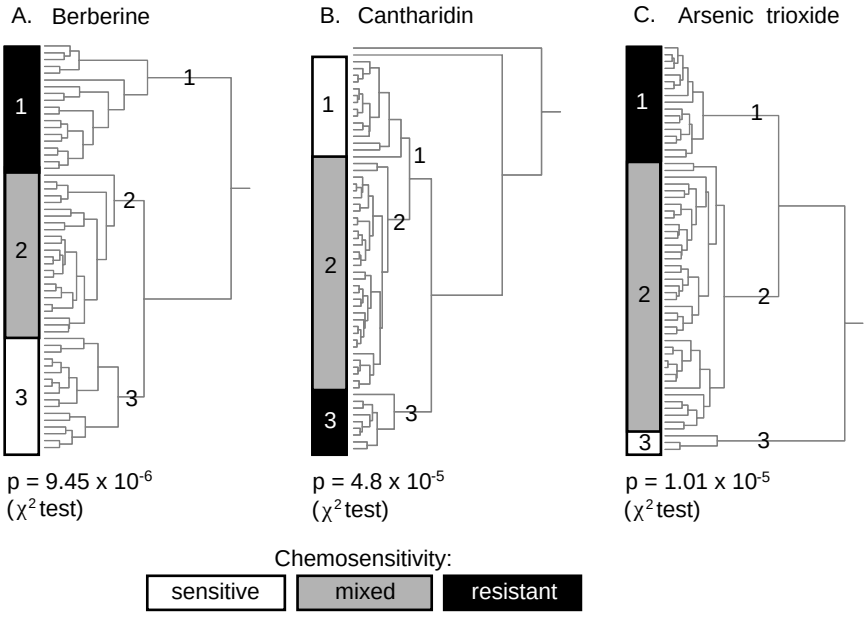

Fig. 2. Hierarchical cluster analysis (complete linkage method) obtained from microarray-based mRNA expression of genes correlating with $\log_{10}IC_{50}$ values for natural compounds derived from traditional Chinese medicine. The dendrograms show the clustering of 60 NCI cell lines for genes identified by COMPARE analyses as being correlated with the $\log_{10}IC_{50}$ values for (A) berberine, (B) cantharidin, and (C) arsenic trioxide. COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the 60 NCI cell lines. Every gene is ranked for similarity of its mRNA expression to the $\log_{10}IC_{50}$ values for berberine, cantharidin, or arsenic trioxide. To derive COMPARE rankings, a scale index of correlations coefficients (R-values) is created. The cluster branches of the dendrograms, which are indicative for sets of genes with similar expressions, were then correlated to the $\log_{10}IC_{50}$ values of the compounds. As shown in the figure, the gene expression profiles are separated in each three gene clusters, which are predictive for sensitivity and resistance to the corresponding natural products.

PPP1R13B, is of special interest among the panel of genes identified by our approach. PPP1R13B plays a central role in the regulation of apoptosis *via* its interaction with the tumor suppressor gene p53. It regulates p53 by enhancing DNA binding activity and trans-activation function of p53 on the promoters of pro-apoptotic genes *in vivo* (Samuels-Lev et al., 2001). A role of PP1 in the repair of UV-induced DNA lesions (Hermann et al., 2002) and the induction of cytosine arabinoside-induced apoptosis was shown

(Wang et al., 2001). It is, therefore, reasonable to hypothesize that PPP1R13B also has specific functions in cantharidin-induced DNA repair and apoptosis. PP1 is one of four major serine/threonine protein phosphatases, and new protein phosphatases are still emerging. They are crucial regulators of many cellular functions by altering the phosphorylation of target proteins. The level of phosphorylation is in a well-controlled balance by the opposing actions of protein kinases and protein phosphatases. The PP1 holoenzyme consists of catalytic and regulatory subunits. Up to now, four catalytic (a, b, c, d) and more than a dozen of regulatory subunits are known. The regulatory subunits modulate the substrate specificity and target the holoenzyme to specific sub-cellular localizations.

A recent study reported the up- or down-regulation of mRNA expression levels of 2087 genes by microarray technology after treatment with cantharidin (Zhang et al., 2004). We compared our set of genes with that of these authors. None of the 21 genes identified in the present study was among the genes identified by Zhang et al. (2004). However, some closely related genes were found with both approaches. Whereas we found the regulatory subunit 13B of protein phosphatase 1 (*PPP1R13B*), Zhang et al. (2004) found regulatory subunit 5 of PP1 (*PPP1R5*). The β2 non catalytic subunit of AMP-activated protein kinase (*PRKAB2*) was identified by us and the γ1 subunit of this enzyme by Zhang and coworkers (2004). In our approach, mRNA expression of cytochrome b5 (*CYB5*) correlated to IC_{50} for cantharidin, Zhang and colleagues (2004) found an altered expression of a DNA sequence which contains the 3' end of the gene for flavohemoprotein b5 and b5R cytochrome b-type NAD(P)H oxidoreductase. Furthermore, heat shock-binding protein 1 (HSBP1) was associated with cantharidin sensitivity in our investigation, while heat shock protein 1 (HSPA1B) was linked to cantharidin treatment in Zhang's study. The small overlap in both microarray-based investigations is surprising at first sight. Two explanations could be attributed to this: First, while our approach focused on the correlation of baseline mRNA expression levels in 60 NCI cell lines and IC_{50} values for cantharidin, Zhang et al. (2004) treated a single cell line (HL60) with a single concentration of cantharidine (25 μM). Those are different biological conditions leading to different gene expression profiles. Second, the selection criteria in our approach were very stringent. We selected 21 out of 9706 genes with the highest COMPARE indices and the smallest probability to correlate erroneously with IC_{50} values for cantharidin by a false discovery rate algorithm. It is probable that many other genes identified by Zhang et al. (2004) also correlate significantly with IC_{50} values for cantharidin in our approach, however with smaller COMPARE correlation coefficients.

Arsenic trioxide

Arsenic is a natural semimetal in soil, water and air. It exists as red arsenic (As_2S_2), yellow arsenic (As_2S_3), white arsenic (As_2O_3, arsenic trioxide), phenylarsine oxide (C_6H_5AsO), and as salts of sodium, potassium and calcium (Miller et al., 2002). Since ancient times arsenic was used as a medicinal agent, i.e., in Greek, Roman, and Chinese medicine (Klaassen, 1996). Arsenic was appreciated as Fowler's Solution for many diseases in the 18th and 19th century, i.e., syphilis, cancer, ulcers, etc. (Haller, 1975). In the 20th century, Paul Ehrlich, the founder of modern chemotherapy, found the arsenical salvarsan which was the standard therapy against syphilis for decades (Chan and Huff, 1997). On the other hand, arsenic compounds can be poisonous (Knowles and Benson, 1983). The revival of arsenic in modern medicine was initiated by Chinese scientists showing dramatic regression rates of acute promyelocytic leukemia (PML) by arsenic trioxide (Shen et al., 1997). These findings were subsequently corroborated in clinical studies in the U.S.A. (Soignet et al., 2001).

Various molecular determinants of the biological effect of arsenic trioxide have been elucidated. It promotes the degradation of the oncogenic fusion protein of the PML and retinoic acid receptor α (RARα) genes in acute promyelocytic leukemia, resulting in induction of cellular differentiation (Chen et al., 1996; Soignet et al., 2001). Apoptosis is selectively induced in malignant cells through enhancement of reactive oxygen species and activation of caspases (Huang et al., 1999; Anderson et al., 2002; Hayashi et al., 2002; Liu et al., 2003). Cells can arrest in the G1 or G2/M phases of the cell cycle after treatment with arsenic trioxide (Liu et al., 2003). Tumor angiogenesis is targeted by arsenic trioxide through inhibition of vascular epithelial growth factor production (Roboz et al., 2000).

As cellular responses of tumor cells to arsenic trioxide seem to be multifactorial, we aimed to get a comprising insight into molecular predictors of sensitivity or resistance to arsenic trioxide (Efferth and Kaina, 2004). For this reason, we mined the NCI's microarray database. Out of 9706 genes, 47 genes whose mRNA expression in 60 tumor cell lines correlated with highest correlation coefficients to inhibition concentration 50% (IC_{50}) values were selected by COMPARE analysis. These genes were subjected to hierarchical cluster analysis and cluster image mapping to reveal, whether the expression profiles of these genes are useful to predict sensitivity or resistance of cell lines to arsenic trioxide.

While the specific functions of the proteins encoded by the 47 genes identified by our approach are different, it is intriguing that many of them are in one way or another involved in apoptosis. This may be taken as a clue that arsenic trioxide impairs the apoptotic machinery and that apoptosis is an important mechanism of arsenic trioxide's cytotoxicity.

Some of the genes identified in the present investigation and genes closely related to them have previously been associated with the action of arsenicals.

We found that the mRNA expression of chemokine-like super family member 4 and growth factor receptor-bound protein 7 correlated with the IC_{50} values for arsenic trioxide. Interestingly, using a microarray approach Wu et al. (2003) found that several cytokines and growth factors were differentially expressed in arsenic-exposed healthy individuals.

The identification of keratin 8 as a predictor of cellular response to arsenic trioxide corresponds to recent findings of Ramirez et al. (2000). These authors observed that arsenite causes DNA-protein cross-links with cytokeratins due to their high content of sulfhydryl groups. Microarray analyses pointed to various keratins (K8, K15, K18, K19) after treatment of cells with arsenic (Yih et al., 2002; Bae et al., 2003).

Another gene found by our approach, thioredoxin reductase I, is inhibited by arsenicals (Lin et al., 2001). Thioredoxin is a regulator of reactive oxygen species that also affects apoptosis by apoptosis-signal-regulating kinase I in an at least partially p53-dependent manner (Ma et al., 2002; Anestal and Arner, 2003).

Our result that aldehyde dehydrogenase 3A2 might contribute to resistance to arsenic trioxide is in accord with the alteration of hepatic mitochondrial aldehyde dehydrogenase activity by sodium arsenate (Fowler et al., 1982).

While a relationship of COX-1 has not been documented previously, COX-2 expression is known to be elevated by arsenite through stimulation of NFκB activity (Tsai et al., 2002). NFκB contributes to arsenic-induced apoptosis (Mathas et al., 2003).

The correlation of mRNA expression of the UBE1 gene in the present investigation implies that the proteasome degradation pathway may play a role for resistance to arsenic trioxide. This view is supported by recent microarray data with arsenic sulfide pointing to the closely related UBE1L gene (Wang et al., 2003).

In conclusion, the data presented here not only provide expression profiles of genes that predict cellular sensitivities and resistance to arsenic trioxide, but may also serve to generate testable hypothesis on molecular mechanisms of cytotoxicity in tumor cells.

Conclusion and perspectives

Using natural products exemplarily taken from medicinal plants, animals, and minerals, we showed how molecular biological and pharmacogenomic approaches can be used to predict sensitivity or resistance of tumor cells

to these agents. This approach also enables the identification of genes possibly involved in molecular pathways responsible for the killing of cancer cells.

The knowledge of molecular mechanisms of cancer cells increased dramatically over the past years. This is a fertile ground to search for the modes of action of natural products. It can be assumed that a wealth of information will be generated in the years to come to unravel the molecular mechanisms of known and novel compounds derived from traditional Chinese medicine. The final goal is to develop novel drugs for cancer treatment. Nevertheless, even after the sequencing of the human genome at the beginning of the new millennium we do not understand the full range of molecular mechanisms of carcinogenesis. Apart from DNA alterations, transcriptional and translational mechanisms (alternative splicing) as well as posttranslational modifications (phosphorylation, glycosylation, acetylation, methylation, ubiquitinylation etc.) must be taken into account elucidating the spectrum of carcinogeneic and tumor promoting mechanisms. The impact of these events and their interaction with natural products needs further to be investigated.

Although many compounds from natural sources have been identified in the past decades and successful protection is possible now, personal assessment, whether a cancer treatment would be successful or not, remains still difficult to predict.

Novel '-omics' technologies (genomics, proteomics, metabonomics) and pharmacogenetics (single nucleotide polymorphisms) may help to define patterns specifically linked to therapy response of tumors and prognosis of survival of patients. On the other hand, it is to be expected that this new dimension of molecular analyses will raise even more questions than it can answer.

A new discipline at the horizon is systems biology. It attempts to integrate the vast amount of data generated by the '-omics' technologies and to generate not only models to describe mechanisms and diseases, but also to develop predictive bioinformatic models for carcinogenic risk assessment at the level of each single individual. This will also be the dawn of a new era in natural product research.

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15 Plant Derived Antimycobacterial Metabolites: An Overview

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1. Introduction

For decades the introduction of very effective drugs has revolutionized the treatment of tuberculosis. In recent years, however, the emerging multiple drug resistant (MDR) and extensively drug-resistant (XDR) strains and HIV epidemics have become a major threat and thus calls for an urgent search for new and effective treatment for this deadly disease. The potential spread of these drug- resistant strains was exemplified by the concern of international health agencies when it was discovered that an individual infected with the extremely drug-resistant tuberculosis (XDR-TB) had flown on two transatlantic flights from the U.S. to Europe and returning via Montreal.

http://news.yahoo.com/s/ap/20070717/ap_on_he_me/tuberculosis_infection

The genus *Mycobacterium* (Mycobacteriaceae) is highly diverse with 85 species known. Some cause human and animal diseases and others are omnipresent in nature. The three most common human mycobacterial diseases are the primary pulmonary disease, tuberculosis, and two skin diseases, leprosy (*M. leprae*) and buruli ulcer (*M. ulcerans*) (Okunade et al., 2004).

Tuberculosis (TB) is one of the oldest and most pervasive diseases in history. TB is caused by *Mycobacterium tuberculosis* (MTB), and to a lesser degree *M. bovis* and *M. africanum*, and continues to be a major disease of global importance infecting at least one third, or two billion, of the world's population (Dye et al., 1999; WHO, 2004). It is a highly infective airborne and chronic bacterial disease usually infecting the lungs, although other organs are sometimes involved. Most healthy individuals are able to control the infection with a vigorous immune response, halting the progression of the disease, but not necessarily eradicating the organism (McKinney, 2000). Most cases are asymptomatic but can be reactivated under certain debilitating circumstances that impair the immune system such as malnutrition, diabetes, malignancy, and especially, AIDS (acquired immunodeficiency syndrome). For example, in sub-Saharan countries over 50% of AIDS related deaths have been attributed to tuberculosis (WHO, 2002). Also in these compromised cases, individuals are also susceptible to infection from *Mycobacterium* species from ubiquitous sources. Late stage AIDS patients are particularly susceptible to the ubiquitous *M. avium* sp. *Avium,* also referred to as the *Mycobacterium avium* complex (MAC). Some members of this group are also known to cause skin ulcers. This was once considered a rare zoonotic nodular wound infection of bird handlers but the disease in immunocompromised persons can result in pulmonary infections, lymphadenopathy and disseminated disease. Pulmonary disease can be caused by other ubiquitous mycobacteria such as *M. fortuitum, M. kansasii, M. intracellularae, M. xenopi* and *M. scrofulaceum* (Czajkowska et al., 2002). Of these, *M. kansasii* disease is associated more closely with levels of immunosuppression and progression of Human Immunodeficiency Virus (HIV) infection than are infections caused by MTB (Canueto-Qintero et al., 2003; Okunade et al., 2004).

In the early 20th century a vaccine made from an avirulent strain of *M. bovis* called Bacille Calmette-Guérin (BCG) was introduced as a prophylactic strategy to fight TB. It is still utilized in many Western and Asian countries, in spite of the fact that its efficacy is variable and immunity tends not to be permanent. In this respect the U.S has opted to detect and treat latent tuberculosis rather than employing this approach which also invalidates the use of the diagnostic skin-test. By the mid 50s the use of Streptomyces-based antibiotics (streptomycin, rifampin) and other chemotherapeutic antimycobacterial agents (pyrazinamide, ethambutol, isoniazid) became epicentral to the treatment of TB. Often used in combination, to prevent the development of drug resistance during prolonged drug therapy, these agents can dramatically reduce the incidence of the disease, particularly when treatment is carefully supervised (Mitnick et al., 2003). Depending upon the severity of the disease, and because of the persistence of sequestered non-replicating bacteria in lung granulomas, successful

treatment usually takes up to six months or more (Boshoff and Barry, 2005).

However, in the early 1990s it was becoming evident that this promising trend was being reversed by a number of factors that continue to promote the emergence of high rates of pulmonary disease and the development of antibiotic resistant strains (Drug-resistant strains—MDR) (Okunade et al., 2004). These factors include their introduction by immigrants from high prevalence nations to developed ones, the active transmission in overcrowded environments such as prisons, hospitals and homeless shelters, particularly in relation to the increase in crack cocaine and intravenous drug use, the HIV/AIDS pandemic (*vide infra)*, in addition to wherever poor nutrition, ineffective diagnosis and poor treatment regimens has promoted their spread (Fatkenheuer et al., 1999). The prevalence of these strains is generally higher in southeastern Asia, sub-Sahara Africa and Eastern Europe. Nevertheless in communities where aggressive control, prevention, disease surveillance and follow-up strategies are employed along with the use of the WHO recommended directly observed treatment short course (DOTS) a reduction in active cases can be achieved.

When ubiquitous mycobacteria are involved (usually in immune compromised individuals such as those with AIDS) treatments are particularly challenging and often require the use of a macrolide antibiotic (either clarithromycin or azithromycin) along with one or more other antimycobacterial agents, such as ciprofloxacin, ethambutol, and rifabutin. Unfortunately, rifabutin and clarithromycin can interact with protease inhibitors, and rifabutin with some non-nucleoside reverse transcriptase inhibitors, making treatment of AIDS patients more difficult (Pablos-Mendez, 1998; WHO, 2003; Okunade et al., 2004).

Despite the availability of effective chemotherapy and moderately protective vaccine, world events and the misuse of these drugs has led to the resumption of TB and the emergence of multiple drug-resistant strains (MDR), which unlike many other bacteria, are a result of step-wise mutations to individual drugs (Pauli et al., 2005). A review on the epidemiology, chemotherapy and mechanism of resistance is presented by Ducati et al. (2006). Clearly there is a critical need to develop new effective agents, a task which could be facilitated by achieving a greater understanding of the molecular mechanisms behind drug activity (McKinney, 2000; Cox et al., 2003; Scherman et al., 2003).

The advancement in bioassay technology over the years has led to the development of relatively time-saving, shorter incubation period assay techniques with lower risk of contamination (Heifets, 1986, 1988; Ashtekar et al., 1987; Chung et al., 1995; Collins and Franzblau, 1997; Ma et al., 2001). A recent mini-review by Pauli et al. (2005) provides a comprehensive

account of the developments in both *in vitro* and *in vivo* antituberculosis bioassays. Most are based on the inhibitory *in vitro* activity against *Mycobacterium* pertaining to C6 (glycolytic) metabolism (Pauli et al., 2005) and cell wall synthesis (Scherman et al., 2003). The type of organisms that are used depends upon the facilities that are available to the investigators. For example, the virulent strain such as *M. tuberculosis* H_{37}Rv, which is representative of drug-resistant clinical isolates, can only be used under strict containment and protective protocols utilizing a biosafety level 3 laboratory (BL-3). An alternative option is to work in a BL-2 cabinet with the slow growing, avirulent strains such as *M. tuberculosis* H_{37}Ra, and the vaccine strains *M. bovis* BCG which are more closely aligned to drug susceptibility and genetic composition than the rapidly growing saprophytic strains like, *M. fortuitum, M. smegmatis, M. avium, M. phlei, M aurum* (Pauli et al., 2005). Of the latter, *M. fortuitum*, is currently the species of choice as an alternative screening model (Gillespie et al., 2001). However, there is also the need to eradicate the tubercle bacilli's anaerobic phase which allows it to persist in a non-replicating state during standard and prolonged TB chemotherapy regimens (Okunade et al., 2004). While assays are known to target this activity (Pauli et al., 2005), they have yet to be applied in any significant way to identifying compounds that could fundamentally shorten the treatment of the disease.

Nonetheless, over the past decade these technologies have led to a surge in the number of compounds that have been added into the scientific literature as possible antimycobacterial agents. Naturally occurring pure compounds as well as extracts from higher and lower forms of plants, microorganisms, fungi, and marine organisms have indicated that inhibitory activity against MTB is widespread in nature. Many compounds isolated using preliminary functional assays have been provided from investigators interested in phytochemical biodiversity. Usually, the potential pharmaceutical worth of these compounds remains unknown since data to show that these compounds are adversely affecting mycobacterial survival mechanisms in humans, or have been derived from medicinal plants is lacking (Okunade et al., 2004).

Hundreds of plants and plant products have been identified as having antimycobacterial properties. A number of comprehensive reviews on antimycobacterial activity of plant species and natural products have appeared in the literature (Newton et al., 2000; Copp, 2003; Okunade et al., 2004; Jachak and Jain, 2006; Copp and Pearce, 2006) in addition to those limited to regional and/or of medicinal plant importance from Ethiopia (Asres et al., 2001), Indian (Rivero-Cruz et al., 2005; Gautam et al., 2007) or the Peruvian Amazon (Lewis et al., 1999; Lewis, 2003). Others are associated with specific types of compounds such as the antimycobacterial activity of plant terpenoids (Cantrell et al., 2001) or antimyco-

bacterial compounds derived from lichens, algae, fungi or marine sources (König et al., 2000; Muller, 2001; Mayer et al., 2007). There continues to be a renewed effort at isolating and assaying compounds from these sources more importantly because natural products are a proven template for the development of new drugs (Cragg et al., 1997; Newman et al., 2000, 2003; Kinghorn, 2001). Nonetheless, challenges exist in making this effort worthwhile in regards to sourcing, the structural complexity of the molecules, the opportunity to synthetic follow up on promising leads, and the need to elicit data on mammalian cytotoxic activity vs their anti-TB potential (Pauli et al., 2005).

While by no means inclusive, this overview will highlight the different structural features of the antimycobacterial natural products primarily from plants, emphasizing the structural diversities of naturally occurring compounds with antimycobacterial properties at minimal inhibitory concentrations (MICs) of ≤100 μg/mL. This will include active compounds from different classes, such as alkaloids, terpenoids, coumarins/chromones, peptides and phenolics. Mention will also be made where necessary of some synthetic analogues with proven antimycobacterial activities especially against certain *Mycobacterium* species associated with AIDS. Even though the focus will mainly be on terrestrial plants, in few cases, mention will be made of compounds from marine organisms as well as from lichens, algae and fungi. Noteworthy among the examples cited are the number of isolates that have been derived from traditional pharmacopeias, often with corollary uses for respiratory illness, tuberculosis, and its related symptoms such as bloody sputa, fever, cough, scrofula (affecting neck lymph nodes) and others associated with tropical skin ulcers that are frequently caused by *M. ulcerans*, *M. leprae,* as well as *ubiquitous Mycobacteria* or related *Nocardia.* Certain related orders in flowering plants, are known to share similar bioreactivities, suggesting a commonality of certain compounds. These are often found in the same phylogenetic group (10 in all) made up of subclasses and/or monophyletic clades. The taxa in Table 1 are presented within phylogenetic groupings (Lewis and Elvin-Lewis, 2003).

Aliphatics and simple aromatics

Two recent reviews (Copp, 2003; Copp and Pearse, 2007) presented a good number of antimycobacterial compounds from these classes of compounds. Simple aliphatic compounds like 3-nitropropionic acid isolated from fungi and gamma lactones and polyacetylenes from a variety of plant sources are covered in these reviews. The polyynes **1-6** from the Devil's Club, *Oplopanax horridus*, inhibit the growth of MTB and. *M. avium* at 10 μg/disk in a disk diffusion assay (Kobaisy et al., 1997). The antibiotic polyyne

Table 1. Traditional sources of potentially useful antitubercular compounds

*PG**	*Family/* ***Compound #***	*Species*	*Common name*	*Tribe/Region*	*Relevant medicinal use*
1	Annonaceae **53**	*Cananga odorata* (Lam.) Hook. f. and Thomson	Dwarf Ylang-Ylang Tree	South East Asia, Thailand, Australia	Respiratory: flowers, asthma, fever (Perry, 1980)
	Annonaceae **51**	*Cliestopholis patens* (Benth.) Engl. and Diels		West Africa	Respiratory: TB in Congo; fever: leaf infusion (Burkill, 1985)
	Annonaceae **7, 55**	*Polyalthia cerasoides* (Roxb.) Benth. and Hook. f. ex Bedd.		South East Asia, Thailand	Respiratory: bark and wood decoction (Perry, 1980)
1	Canellaceae **98**	*Warburgia ugandensis* Sprague		East Africa	Respiratory: inner bark; also for pain; Fever (malaria) (Kokwaro, 1976).
1	Lauraceae **91, 92**	*Laurus novocanariensis* Rivas Mart., Lousa	Laurel oil	Madiera Islands	Antiinfective: Oil (Rivera and Obon, 1995)
	Piperaceae **14-20**	*Piper sarmentosum,* Roxb.		SE Asia	Respiratory: rootlets chewed for coughsand asthma (Perry, 1980)
		P. sanctum (Miq.) Schltdl. ex C. DC.		MesoAmerica, Mexico N.	Respiratory: asthma, T.B (Mata et al., 2004); Medicinal: rootscarminative
		P. pedicellatum C. DC.		Thailand	(Rukachaisirikul et al., 2004)
	Acanthaceae **36**	*Strobilanthes cusea* (Nees) Kuntze		China/Taiwan, India	Fever: roots and leaves (*A Barefoot Doctors Manual.* Running Press ISBN

Contd.

Table 1 continued

PG*	Family/ **Compound #**	Species	Common name	Tribe/Region	Relevant medicinal use
					0-914294-92-X) Respiratory: cough (Sivarajan and Balachandran, 1994)
	Plumbaginaceae **142**	*Plumbago zeylanica* L.	Citrakah	India	Skin: as irritant (Sivarajan and Balachandran, 1994)
4	Clusiaceae **78**	*Calophyllum lanigerum* Miq.	Mangosteen	Malaysia	Unknown; antiinfective: (*C. inophyllum L.*) for skin ulcers (Perry, 1980)
	Clusiaceae **86, 85**	*Garcinia mangostana* L.	Mangosteen	SE Asia	Fever: leaves; antiinfective: root decoction for skin infections, ulcers (Perry, 1980)
	Euphorbiaceae **76**	*Celaenodendron mexicanum* Standl.			Unknown
	Euphorbiaceae **114, 115**	*Croton kongensis* Gagnep.		China	Unknown, (*C. tiglium* L): seeds Respiratory
	Euphorbiaceae **133**	*Euphorbia lagascae* Spreng.		SE Europe	Skin: ulcers, cancers, tumors, warts (Hartwell, 1969)
	Euphorbiaceae **99-104**	*Pedilanthus tithymaloides* (L.) Poit.	Slipper flower, Japanese Pointsetta	SE Asia, Oceania, Thailand, South and MesoAmerica	Medicinal: skin lesions (caustic) (Johnson, 1999)

Contd.

Table 1 continued

PG*	*Family/* ***Compound #***	*Species*	*Common name*	*Tribe/Region*	*Relevant medicinal use*
	Euphorbiaceae **116** **113**	*Sapium haematopsermum* Müll. Arg. *S. indicum*			Unknown, (*Sapium sebiferum* (L.) Roxb) Antiinfective: skin ulcers
	Fabaceae **171-173**	*Bauhinia purpurea* Wall.	Kancanarah	Thailand India	Respiratory: cough; skin: ulcers, leprosy; glandular swellings
	Fabaceae **57-67**	*Derris indica* (Lam.) Bennet		East and SE Asia	Antiinfective: skin ulcers (Sivarajan and Balachandran, 1994)
	Fabaceae **56**	*Glycyrrhiza inflata* Batalin	Sinkiang Licorice, Gancao	China	Respiratory: TCM—roots for cough, asthma
	Flacourtiaceae **105-110**	*Casearia grewiifolia* Vent.		Thailand	Fever: bark and flower decoctions (Copp and Pearce, 2006)
	Juglandaceae **143**	*Engelhardia roxburghiana* Wall.		Taiwan	Unknown
4	Moraceae **71**	*Artocarpus rigidus* Blume	Peruput	SE Asia, Malaysia, Thailand	Antinfective: wounds (antiinfective); Fever: (*A. rubrovenius* Warb.) (Perry, 1980)
	Polygonaceae **133-135**	*Ruprechtia triflora* Griseb.		Argentina, Brazil, Paraguay	Unknown

Contd.

Table 1 continued

PG*	Family/ **Compound #**	Species	Common name	Tribe/Region	Relevant medicinal use
	Rhamnaceae **138, 139**	*Colubrina retusa* (Pittier) R.S. Cowan	Buckthorn		Unknown: other species Skin Diseases (Perry, 1980; Moerman, 1999)
	Zygophylliaceae **153**	*Larrea divaricata* Cav.	Creosote Bush	Mexico, Argentina	Inflammation: anti-rheumatic (Pedernera et al., 2006)
5	Combretaceae **161**	*Combretum molle* R. Br. ex G. Don		East Africa, Upper Volta	Respiratory: leaf. (Kokwaro, 1976) Respiratory: decoction leafy twigs bronchitis (Burkill, 1985)
	Rutaceae **22**	*Amyris elemifera* L.		SE, U.S.A., MesoAmerica, Caribbean	Respiratory: influenza; fever (Johnson, T CRC Ethnobotany Desk Reference)
	Rutaceae **37-40, 79, 80**	*Clausena excavata* Burm. f.		SE Asia, India, Thailand, China	Fever: root; antiinfective: skin ulcers (*C. anisum-olens* Blanco.) Merr. decoction ofroot and fruit: cough with fever (Perry, 1980)
5	Rutaceae **30-34**	*Evodia ruticarpa* (A. Juss.) Benth.	Fructos Evodiae Rutaecarpae	China, Korea	Medicinal: traditional Chinese Medicine (TCM); fruit for epigastric pain, gastrointestinal complaints (Bensky and Gamble, 1993)

Contd.

Table 1 continued

*PG**	*Family/* ***Compound #***	*Species*	*Common name*	*Tribe/Region*	*Relevant medicinal use*
5	Rutaceae **26-29**	*Galipea officinalis* J. Hancock (correct citation as *G. cusparia* A. St.-Hil. ex DC)	Angostura	Venezuela	Fever: bark
5	Rutaceae **23-25**	*Lunasia amara* Blanco		SE Asia, New Guinea	Medicinal: topical antiinfectant, tropical ulcers (Prescott et al., 2007)
5	Rutaceae **35**	*Mitracarpus scaber*, Zucc [syn. *M. villosus* (Sw.) DC]		West Africa	Medicinal, antiinfective, leprosy (Dalziel, 1936)
6	Apocynaceae **47**	*Tabernaemontana citrifolia* L.	Milkwood	West Indies MesoAmerica	Fever: latex (Morton, 1981)
6	Asclepiadiaceae **41-43**	*Cryptolepis sanguinolenta* (Lindl.) Schltr.		W. Africa	Fever (Iwu et al., 1999)
6	Bignoneaceae **88**	*Incarvillea dissectifoliola* Q.S. Zhao		China	Unknown
6	Bignoniaceae **144**	*Tabebuia* Gomes ex DC.		South America	Many species medicinal e.g., *T. avellanedae* (Pau d'Arco) for fever, skin ulcers, pain, anti-infective.

Contd.

Table 1 continued

PG*	*Family/* ***Compound #***	*Species*	*Common name*	*Tribe/Region*	*Relevant medicinal use*
6	Ebenaceae **147**	*Euclea natalensis* A. DC.		South Africa	Respiratory: cough, TB. Arnold and Gulumian, 1984
6	Lamiaceae **133**	*Ajuga remota* Wall. ex Benth.		China, E. Africa	Leaves: Fever: (Kareru et al., 2007) Antiinfective: (Glover et al., 1966) Skin: ulcers etc. (Lindsay and Hepper, 1978)
6	Lamiaceae **111**	*Leucas volkensii* Gürke		Kenya	Unknown, (*L. martinsensis* (Jacq.) Ait.f: Respiratory, fever, wounds (Burkill, 1985)
6	Lamiaceae **112**	*Salvia multicaulis* Vahl		SW Asia	Unknown
6	Primulaceae **141**	*Primula obconica* Hance	German primrose	China	Skin irritant, allergen
6	Rubiaceae **111, 130-132**	*Morinda citrifolia* L.	Noni, Indian Mulberry	Polynesia, Phillipines	Antiinfective: skin ulcers, immature fruit TB, respiratory (Saludes et al., 2002)
6	Rubiaceae **145, 146**	*Prismatomeris fragrans* E.T. Geddes		Thailand	*P. tetrandra* for bronchitis (Perry, 1980)
6	Solanaceae **140**	*Physalis angulata* L.		Amazonia, W Africa	Anti-inflammatory Respiratory; (Burkhill, 1997 v4)

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Table 1 continued

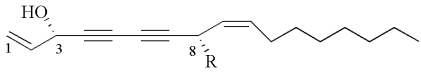
*PG**	*Family/* ***Compound #***	*Species*	*Common name*	*Tribe/Region*	*Relevant medicinal use*
6	Solanaceae **21**	*Solanum sodomaeum* Drege in DC.	Apple of Sodom, Nightshade	North Africa	Respiratory: cough (Uphof, 1968), tuberculosis
6	Theophrastaceae **123**	*Clavija procera* B. Ståhl		Aguaruna Jivaro: Peru	Antiinfective: skin ulcer (Rojas et al., 2006).
6	Verbenaceae **119-120**	*Lantana hispida* Kunth		Mexico	Respiratory: TB bronchitis, cough. (Jimenez-Arellanes et al., 2007)
7	Apiaceae **6, 81, 82**	*Anethum graveolens* L.	Dill	Cosmopolitan	Respiratory: seed or Plant decoction-Grippe in babies (Chevallier, 1996) aromatic herb and spice in cookery (Stavri and Gibbons, 2005)
7	Apiaceae **77**	*Peucedanum ostruthium* (L.) W. Koch	Masterwort	Eurasia	Respiratory: root expectorant (Chiej, 1984)
	Araliaceae **1-5**	*Oplopanax horridus* Torrey and Gray ex. Miquel	Devil's Club	NW Amerindians and neighboring First Nation tribes	Respiratory: bark and roots for TB. Respiratory illnesses, bloody sputa, (Kobaisy et al., 1997; Moerman, 1999; Lantz et al., 2004)
7	Asteraceae **96, 97**	*Inula helenium* L.	Elecampane Inula	NE Amerindians and neighboring First Nation tribes	Respiratory: root and/or leaf decoctions for tuberculosis, other respiratory conditions. (Moerman, 1999)

Contd.

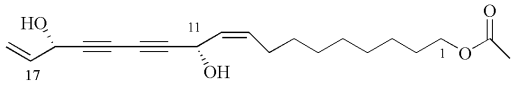
Table 1 continued

PG*	Family/ **Compound #**	Species	Common name	Tribe/Region	Relevant medicinal use
7	Asteraceae **68-70**	*Haplopappus sonoriensis* (A. Gray) S.F. Blake	Goldenbush, Common cocklebur	Mexico	Unknown: other species herbal teas and medicinal
7	Asteraceae **152**	*Iostephane heterophylla* (Cav.) Benth ex Hemsl	Manso	Mexico	Respiratory: root decoction (Sandoval et al., 2005)
7	Valerianaceae **117, 118**	*Valeriana laxiflora* DC.		Chili	Unknown: some NA species used for respiratory (*V. sitchenensis* Bong. Root decoction for TB), skin ulcers by Amerindians and First Nationpeoples (Moerman, 1999)
9	Liliaceae **44, 45**	*Allium neapolitanum* Cirillo	White Garlic	S. Europe, N. Africa, Middle East	Respiratory: colds, sore throats and coughs
9	Liliaceae **83, 84**	*Chlorophytum inornatum* Ker. Gawl		India	Unknown: other species: antiinfective (Sivarajan and Balachandran, 1994)

*Phylogenetic group. # Compound number.



1 R = H
2 R = OH
3 R = OH; 1, 2 dihydro



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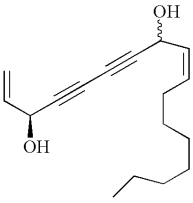
5 17, 18 dihydro



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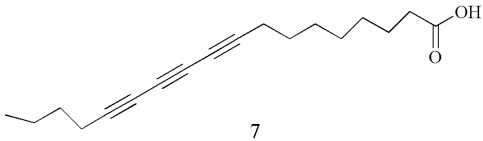

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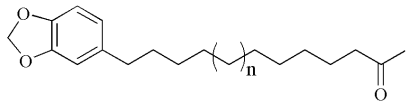
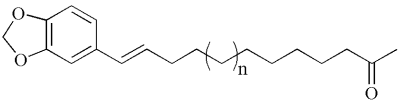
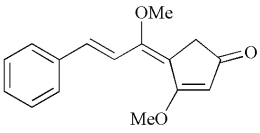


8 n = 3
9 n = 5



10 n = 5



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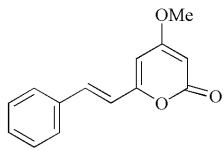

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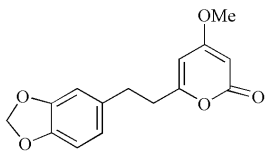

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Fig. 1. Aliphatics and simple aromatics.

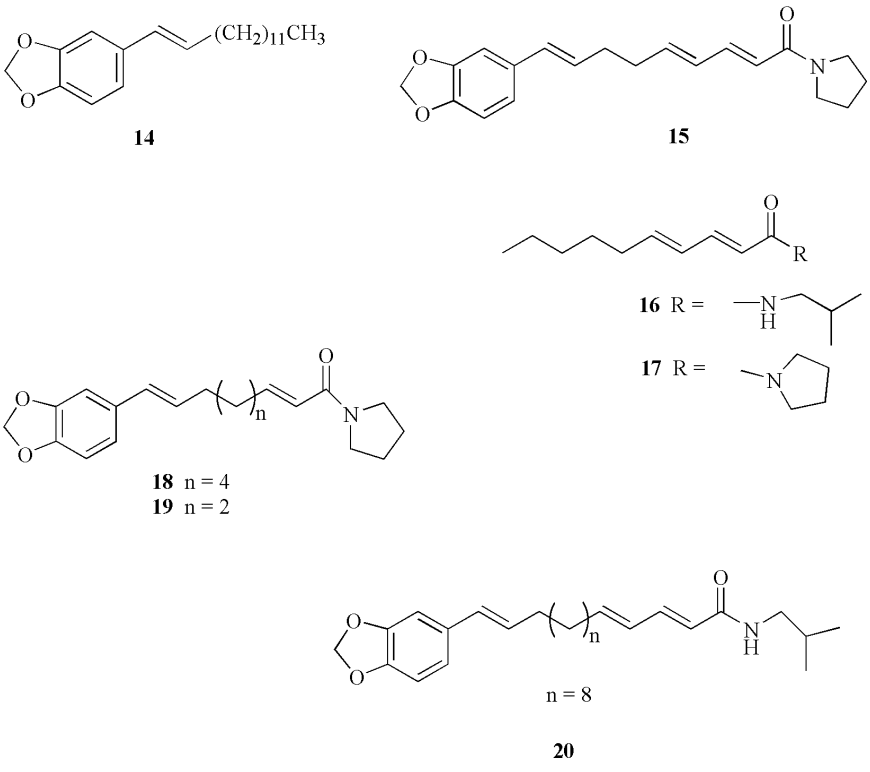


Fig. 1 continued. Aliphatics and simple aromatics.

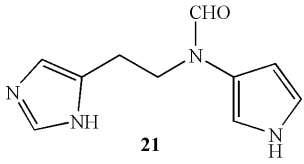
falcarindiol **6** was also isolated from Dill (*Anethum graveolens*) and shown to exhibit strong activity against a panel of fast growing mycobacteria species *M. fortuitum, M. phlei, M. aurum, M. smegmatis* and *M. abscessus* cultured on Columbia blood agar (Oxoid) and supplemented with 7% defibrinated horse blood (Oxoid). The MIC values range from 2-4 µg/mL. (Stavri and Gibbons, 2005). Octadecatrynoic acid **7** from the roots of *Polyathia cerasoides* showed antimycobacterial activity against the drug resistant strain MTB H_{37}Ra in the Microplate Alamar Blue Assay (MABA) with MIC value of 6.25 µg/mL (Kanokmedhakul et al., 2007).

Phytochemical studies of fresh roots of *Piper sarmentosum* and leaves of *P. sanctum* resulted in the isolation of amides and aromatic alkenes with antimycobacterial activities. Compounds **8-13** from *P. sanctum* significantly inhibit the growth of drug sensitive strain of MTB (H_{37}Rv, ATTC27294) in MABA (Mata et al., 2004). Compound **13** was the most potent with MIC value of 4 µg/ml but was also the most cytotoxic of the compounds. Both

tetradecane derivative **8** and the hexadecane derivative **9** have MIC value of 6.25 µg/mL, z-piperolide **11** has an MIC of 64 µg/mL and the other two compounds, **10** and **12,** both have MIC of 32 µg/mL Using the same assay, the amides **15-20** as well as the aromatic alkene **14** from *P. sarmentosum* exhibited antimycobacterial activity against MTB H_{37}Ra with MIC values ranging from 25- 50 µg/mL (Tuntiwachwuttikul et al., 2006).

2. Alkaloids

Many plant alkaloids are well known therapeutic agents. Some of the known naturally-occurring alkaloids and analogs have been found to possess antimycobacterial activities. The indoloquinazolinone alkaloid tryptanthrin **36** from the Chinese/Taiwanese medicinal plant *Strobilanthes cusia*, found in a number of preparations for respiratory infections, was studied extensively as result of its potent activity against MTB (H_{37}Rv) (MIC 1 µg/mL) and MAC (MIC 2 µg/mL) in Bactec studies, and *M. smegmatis* (MIC 4 µg/mL) in agar-dilution test (Mitscher and Baker, 1998). Simple plant alkaloids with antimycobacterial activities include pyrrole alkaloid solsodomine **21** isolated from *Solanum sodomaeum* (El Sayed et al., 1998). This compound was active against the AIDS pathogen *Mycobacterium intracellulare* (MIC 10 µg/mL). The oxazole alkaloid texalin **22** isolated from *Amyris elemifera* showed a modest activity against MTB H37Rv and two other related species, *M. kansasii* and *M. avium* with MIC value of 25 µg/mL in the Middlebrook 7H11 agar medium and Bactec 460-TB radiometric assay (Rastogi et al., 1998). Quinoline alkaloids **23-25** from *Lunasia amara* inhibit the MTB H_{37}Rv in a Bactec 460 assay. Both **23** and **24** have MIC value of 16 µg/mL while 24 have MIC value of 33 µg/mL (Aguinaldo et al., 2007). Ethanol extracts of Angostura back, *Galipea officinalis* afforded quinoline alkaloids **26-29** that were active against 10 strains of MTB with MICs ranging from 6.25-50 µg/ml (Houghton et al., 1999). The quinolones **30-34** isolated from *Evodia ruticarpa* have also been reported to have antimycobacterial activities. MICs range from 2-32 µg/mL against a panel of fast growing mycobacteria including *M. fortuitum*, *M. smegmatis* and *M. phlei* (Adams et al., 2005) The azaanthraquinone **35** from *Mitracarpus scaber* is a good inhibitor of *M. intracellulare* (MIC 6.25 µg/mL, Okunade et al., 1999). The carbazoles **37-40** obtained from the rhizomes and roots of *Clausena excavata* (Rutaceae) all have modest activity against MTB (MIC 50-100 µg/mL) (Sunthikawinsakul et al., 2003). The indoloquinoline alkaloid **41-43** from *Cryptolepis sanguinolenta* showed activity against *M. fortuitum* (Gillespie et al., 2001). The hydrochloride of cryptolepine **41,** a well known DNA intercalator, also has a good activity against a related *M. bovis* (MIC 12.5 µg/mL) (Gibbons et al., 2003). **42** has an MIC value of 31 µg/mL against *M. fortuitum* while the dimer **43** has a value of 6.25 µg/



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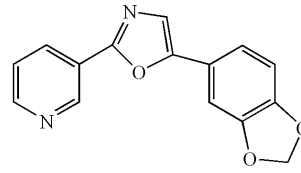

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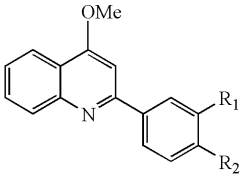
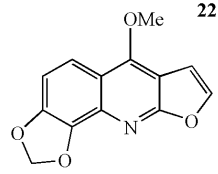


23 $R_1 = R_2 = H$
24 R_1, R_2 = -O-CH_2-O-



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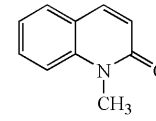

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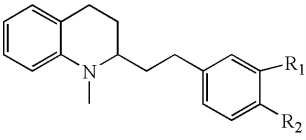
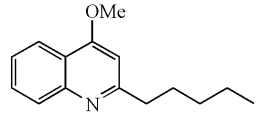


28 $R_1 = R_2$ = OMe
29 R_1, R_2 = -O-CH_2-O-



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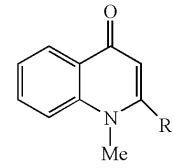
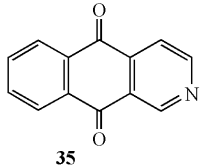


30 R = n-$C_{11}H_{23}$
31 R = $C_{11}H_{21}$ D6
32 R = $C_{13}H_{24}$ D8
33 R = $C_{13}H_{22}$ D4,7
34 R = R = $C_{15}H_{28}$ D6,9



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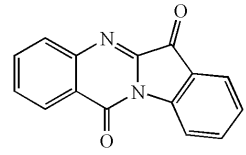

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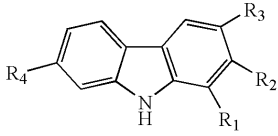

37 $R_1, R_2, R_4 = H, R_3 = CHO$
38 $R_1, R_2, R_4 = H, R_3 = CO_2CH_3$
39 R_1, = H, R_2, = OH, R_3 = CHO, $R_4 = OCH_3$
40 R_1, = H, $R_2, R_4 = OCH_3, R_3 = CO_2H$,

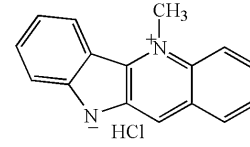

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Fig. 2. Alkaloids.

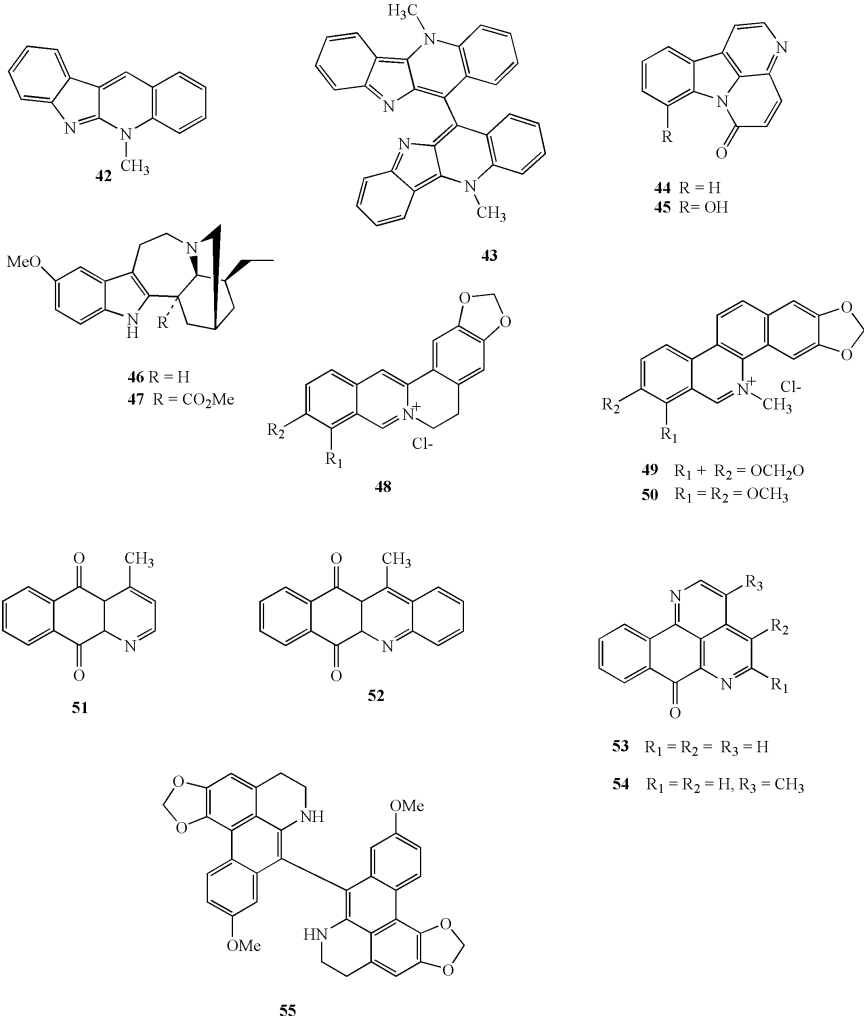


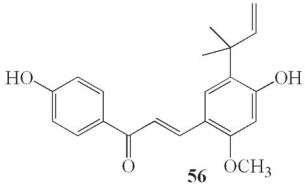
Fig. 2 continued. Alkaloids.

mL. Canthin-6-one **44** and its 6-hydroxy derivative **45** isolated from *Allium neapolitanum* significantly inhibits the growth of a panel of fast-growing Mycobacterial species of *M. fortuitum*, *M. smegmatis*, *M. phlei* and *M. abcessus*. MICs range from 8-32 µg/mL. (O'Donnell and Gibbons, 2007). Other structurally complex indole alkaloids with modest antimycobacterial activities (MICs 50-100 µg/mL) include ibogaine **46** and voacangine **47** isolated from *Tabernaemontana citrifolia* (Rastogi et al., 1998). The bis-benzylisoquinoline alkaloid berberine **48** is found in numerous genera

including species of Coptis and Berberis (Hsieh et al., 2007) inhibits the growth of *M. smegmatis* at MIC of 25 µg/ml and *M. intracellulare* (MIC >100 µg/ml) (Okunade et al., 1994; Mitscher and Baker, 1998; Gentry et al., 1998). The structurally related benzo[c]phenanthridine alkaloid chlerythrine **49** and sanguinarine **50** are other examples that demonstrated appreciable activity against MTB H_{37}Rv, *M. avium*, *M. bovis* BCG and *M. smegmatis* with MIC of 12.5 µg/mL. It is believed that the iminium ion improves the lipophilicity and hence increases the bioavailability of the alkaloids to the organisms. Synthetic analogs **52** and **54** of the naturally occurring cleistopholine **51** (from *Cleistopholis patens*, Annonaceae) and sampangine **53** (from *Cananga odorata*, Annonaceae) strongly inhibit the AIDS pathogen *M. intracellularae* (MIC **52**, 1.56 µg/mL; **54**, 0.39 µg/mL equal to or less than those recorded for the control drug rifampin (Peterson et al., 1992). The dimeric aporphine alkaloid from bidebiline E **55** from *Polyalthia cerasoides* also showed antimycobacterial activity against MTB H_{37}Ra (MIC 6.25 µg/mL). (Kanokmedhakul et al., 2007).

3. Flavonoids, coumarins, chromones and xanthones

These plant pigments are very widely distributed in the plant kingdom and have a wide range of biological activities. A good number of them have been reported to exhibit a modest activity against MTB (Copp, 2003; Okunade et al., 2004 and Copp and Pearce, 2006). The structure-activity relationships of a series of non-natural flavonoids were reported by Lin et al. (2002). They observed that chalcones are more active than the flavones and flavanones probably because they are more geometrically constrained. The authors also reported that the 2′-hydroxy substitution of chalcone and flavonoids enhances the antimycobacterial activity. The licochalcone **56** isolated from the root of Chinese licorice *Glycyrrhiza inflata* (Friis-Moller et al., 2002) was very active against MTB (MIC 5-10 µg/mL), *M. bovis* (MIC 10-20 µg/mL) and *M. bovis* BCG (MIC 5-10 µg/mL) but less potent towards *M. intracellulare* (MIC 20-80 µg/mL) and *M. avium* (MIC >80 µg/mL). A series of flavonoid compounds **57-67** from *Derris indica* exhibited antimycobacterial activity against MTB H_{37}Ra with MIC values between 6.25 and 100 µg/mL (Koysomboon et al., 2006). The flavonols **68-70** isolated from *Haplopappus sonoriensis* (Asteraceae) inhibited the growth of MTB H37Rv by 33, 98 and 48% respectively at a concentration of 100 µg/mL (Murillo et al., 2003). The flavonoid artonin F **71** was the most potent of the compounds isolated from *Artocarpus rigidus* that showed antimycobacterial activity against MTB H_{37}Ra with MIC 6.25 µg/mL (Namdaung et al., 2006). Others compounds from this plant with moderate activities in Microplate Alamar Blue assay, include flavonoids **72, 73** (MIC 25 and 50 µg/mL respectively), chromone artorigidusin**74** and xanthone **75** both



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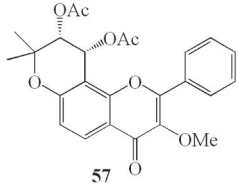

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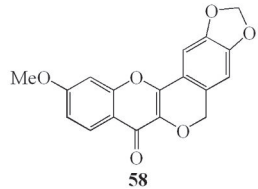

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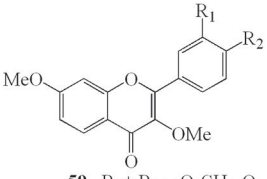
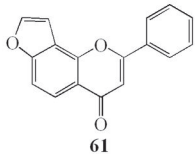


59 $R_1 + R_2 = O\text{-}CH_2\text{-}O$
60 $R_1 = R_2 = H$



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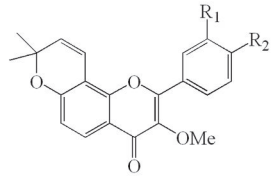
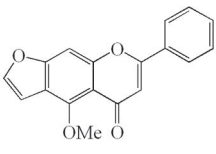


62 $R_1 + R_2 = O\text{-}CH_2\text{-}O$
63 $R_1 = R_2 = H$



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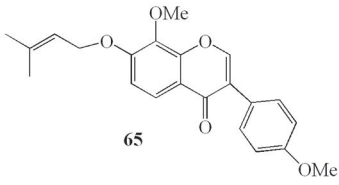

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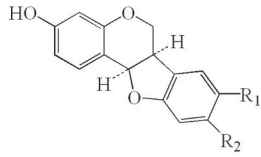
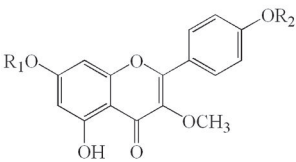
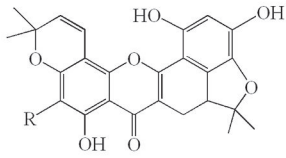


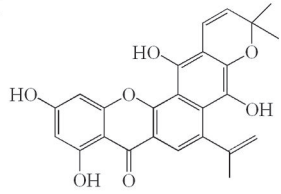
66 $R_1 + R_2 = O\text{-}CH_2\text{-}O$
67 $R_1 = R_2 = CH_3$



68 $R_1 = R_2 = CH_3$
69 $R_1 = H, R_2 = CH_3$
70 $R_1 = CH_3, R_2 = H$



71 $R = -CH_2CH{=}C(CH_3)_2$
72 R = H



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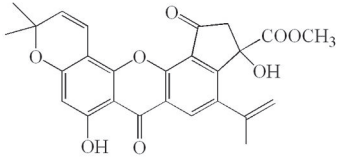

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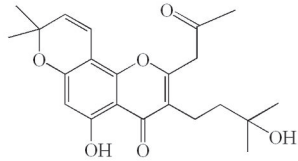

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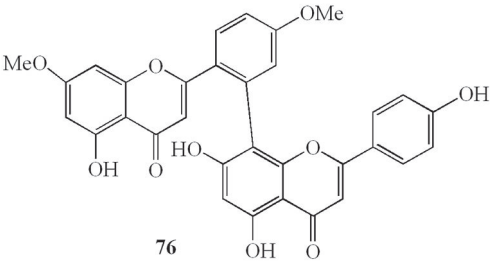
Fig. 3. Flavonoids, coumarins, chromene and xanthone.



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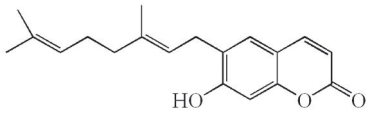

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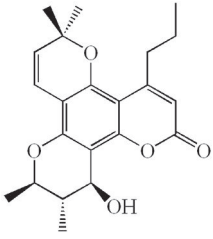

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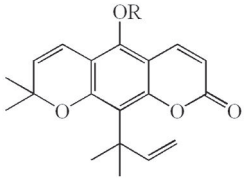
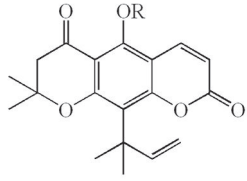


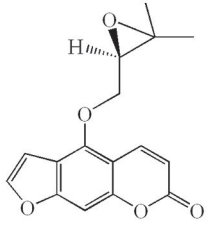
79 R = CH_3

80 R = H



81 R = H

82 **R** = CH_3



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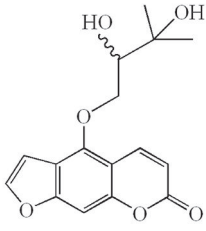

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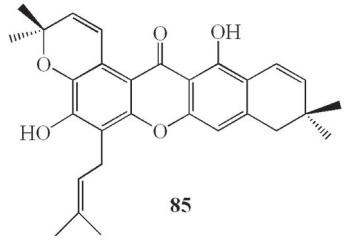

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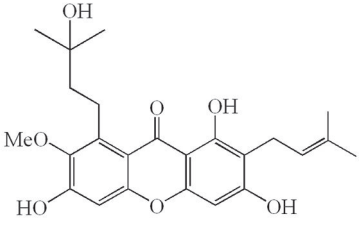


86

Fig. 3 continued. Flavonoids, coumarins, chromene and xanthone.

with MIC value of 12.5 µg/mL. **71-73** also show antiplasmodial activities and all five compounds (**71-75**) are cytotoxic to one or two cancer cell lines but **71** is not toxic at 20 µg/mL. Biflavonoid **76** previously isolated from the Mexican plant *Celaenodendron mexicanum* (Castañeda et al., 1992) showed a mild activity against MTB $H_{37}Rv$ (MIC 64 µg/mL) (Rivero-Cruz et al., 2005). Coumarins are also known to possess anti TB activities. The anti HIV agent ostruthin **77** from *Peucedanum ostruthium* (Apiaceae) with the prenyl group at C-6 demonstrates potent activity against different strains of the rapidly growing mycobacteria, *M. aurum, M. fortuitum*, *M. phlei* and *M. smegmatis,* with MIC values of 3.4-6.7 µM, analogous to the MIC value obtained for the control isoniazid (Schinkovitz et al., 2003). The presence of the prenyl group appears to be important to the activity of the coumarins, since it increases the lipophilicity and hence the bioavailability to the organism as earlier observed in some sesquiterpenes (Cantrell et al., 2001). The pyranocoumarin (+) calanolide **78** also known as an anti HIV agent from *Calophyllum lanigerum* exhibits a strong activity towards MTB $H_{37}Rv$ (MIC 3.3 µg/mL) and an array of drug resistant strains with MIC values ranging from 8-16 µg/mL. Preliminary mechanistic studies showed that this compound swiftly inhibits RNA and DNA synthesis followed by an inhibition of protein synthesis. Other analogs of this compound are also shown to exhibit similar antimycobacterial activity (Xu et al., 2004). The pyranocoumarins dentatin **79** and nor-dentatin **80** from *Clausena excavata* are modestly active against MTB $H_{37}Ra$ (MIC 50, 100 µg/mL respectively). The related natural xanthoxyletin **79B** was inactive and hence it is believed that the prenyl at the C-8 position is essential for the antimycobacterial activity. The 5-O prenylated furanocoumarins **81, 82** from dill—*A. graveolus* also demonstrated moderate activity against a panel of rapidly growing mycobacteria, *M. phlei*, ATCC 11758, *M. aurum* Pasteur Institute 104482, *M. smegmatis* ATCC 14468 and *M. abscessus* ATCC 19977 with MIC values ranging from 32-64 µg/mL (Stavri and Gibbons, 2005). The homo-isoflavanone, 3-(4-methoxybenzyl)-7,8-methylenedioxy-chroman-4-one **83** and the dihydrobenzopyran **84** isolated from *Chlorophytum inornatum* exhibit modest antimycoabacterial activities. **83** was active against *M. phlei* (MIC 16 µg/mL) and *M. aurum* (MIC 32 µg/mL) while **84** was only active against *M. aurum* (MIC 64 µg/mL) (O'Donnell et al., 2006). A series of prenylated antimycobacterial xanthones typified by compounds **85, 86** have also been reported from *Garcinia mangostana* with MICs ranging from 6.25-25 µg/mL (Suksamrarn et al., 2003).

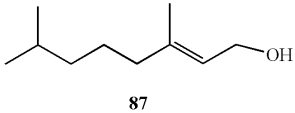
4. Terpenoids

This group is subdivided into monoterpenes, sesquiterpenes, diterpenes and triterpenes. The antimycobacterial activity of this class of compounds

has been comprehensively reviewed by Cantrell et al., 2001; Copp, 2003 and Copp and Pearce, 2006. No less than 300 examples of naturally occurring terpenoids were reported to have moderate to high antimycobacterial activity against MTB and more compounds are being added to the literature. A few examples of the subdivisions will be discussed here and the reader is directed to the earlier reviews. A few monoterpenes have been shown to possess antimycobacterial properties. This includes geraniol **87** which is moderately active against MTB (MIC 64 µg/mL) (Rajab et al., 1998). Chen et al. (2003) reported that the monoterpene dissectol **88** isolated from *Incarvillea dissectifoliola* exhibited mild activity towards MTB 90-221387 in a disk diffusion assay. Also the bornyl ester **89** from *Piper* aff. *pedicellatum* exhibited a significant activity against the H_{37}Ra strain of MTB H_{37}Ra (MIC 25 µg/mL) (Rukachaisirikul et al., 2004).

Sesquiterpenoids particularly the sesquiterpene lactones exhibit a wide range of biological activities and very many of them have been shown to have highly significant antimycobacterial activities. Farnesol **90** have been reported to show activity against MTB H_{37}Rv with MIC of 8 µg/mL. Sesquiterpenoid lactones of the germacranolide, guaianolide and eudesmanolide are known to be anti-tubercular with MICs ranging from 2 to 128 µg/mL (Cantrell et al., 2001). The germacranolide costunolide **91** and dehydrocostuslactone **92** which respectively constitutes 3.8% and 1.5% of the oil expressed from the ripe fruit of *Laurus novocanariensis* (Laurel oil) exhibit the growth of an array of drug-resistant strains of MTB H_{37}Ra with a MIC of 6.25 and 12.5 µg/mL, respectively (Luna-Herrera et al., 2007). Using the fluorometric Alamar Blue microassay, the authors showed that the two compounds acted synergistically amplifying the antimycobacterial activity in contrast to either used alone. Other plant derived germacronolide sesquiterpenes like parthenolide **93**, epoxy costunolide **94** and santamarine **95** are all moderately active against MTB H_{37}Rv (MIC 16, 64, 64 µg/mL, respectively) (Fischer et al., 1998). The eudesmanolide sesquiterpene lactones alantolactone **96** and isoalantolactone **97** were isolated from the root extract of *Inula helenium*. Both compounds exhibited antimycobacterial activity against MTB H_{37}Rv with MIC values of 32 µg/mL. It has been suggested that the α-methylene-γ-lactone ring as well as the presence of an additional alkylating site together with moderate to high lipohilicity enhance the *in vitro* activity of these group of compounds (Fischer et al., 1998 from Copp, 2003; Cantrell et al., 1999a, 2001). The East African plant *Warburgia ugandensis* afforded the antimycobacterial sesquiterpene lactone muzigadial **98** that exhibits moderate activities towards a panel of antimycobacterial strains, *M. avium* and *M. phlei, M. fortuitum* and *M. smegmatis* (MIC 16-64 µg/mL) (Wube et al., 2005).

From the white latex of the Thai plant *Pedilanthus tithymaloides* were recently isolated six new poly-O-acylated jatrophane diterpenoids **99- 103**



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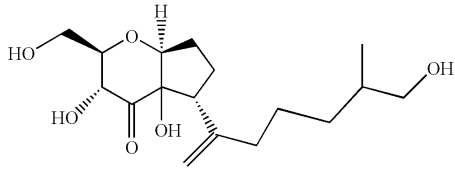

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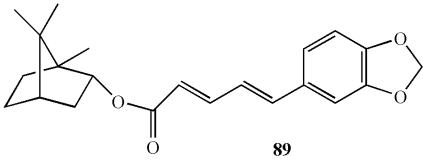

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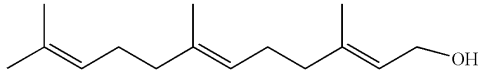

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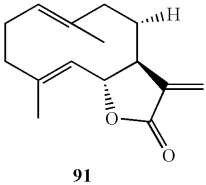

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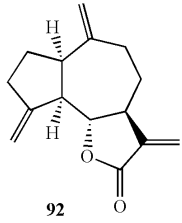

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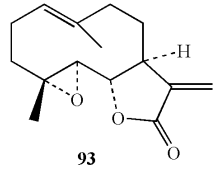

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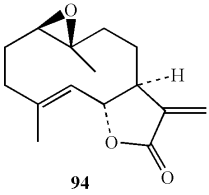

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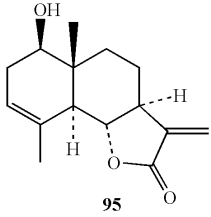

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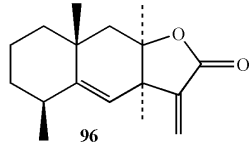

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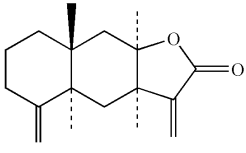

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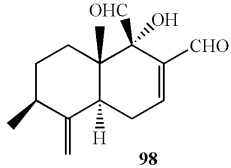

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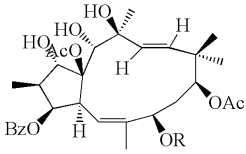
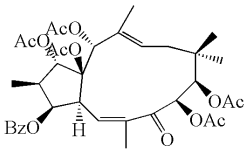
Fig. 4. Terpenoids.



99 R = Bz
100 R = H



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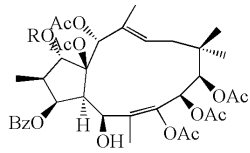
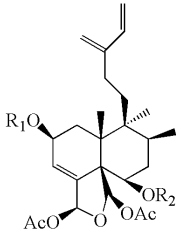
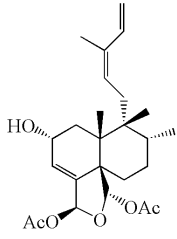


102 R = H
103 R = Ac





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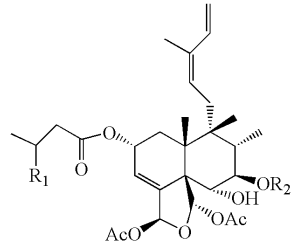
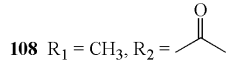
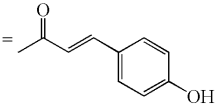


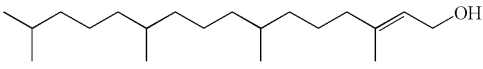
104 $R_1 = (CH_3)_2CHCH_2CO$, $R_2 = CH_3$
105 $R_1 = CH_3CH_2CH(CH_3)CO$, $R_2 = CH_3$
106 $R_1 = CH_3CH_2CH(CH_3)CO$, $R_2 = OH$



108 $R_1 = CH_3$, $R_2 =$



109 $R_1 = H$, $R_2 =$



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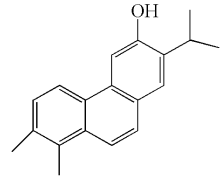

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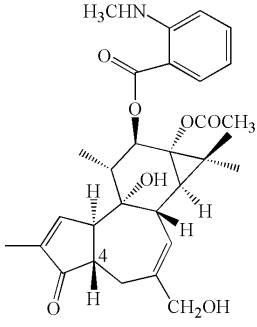

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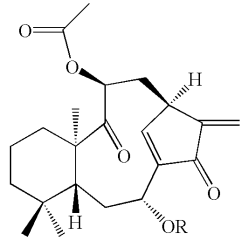
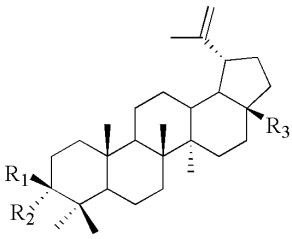
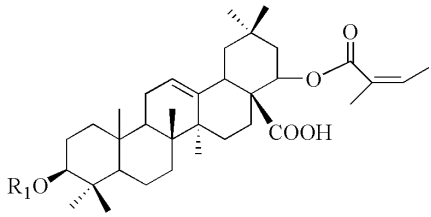


113 R = Ac
114 R = H

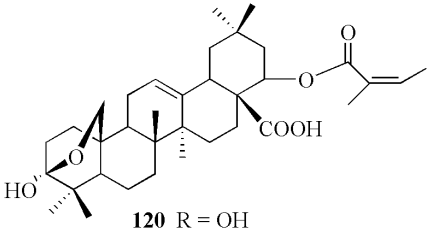
Fig. 4 continued. Terpenoids.



115 R_1 = OH, R_2 = H, R_3 = Me
116 R_1 = OH, R_2 = H, R_3 = COOH
117 R_1 = H, R_2 = OH, R_3 = Me

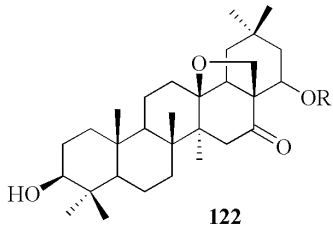


118 R_1 = $COCH_3$
119 R_1 = H



120 R = OH

121 R = H



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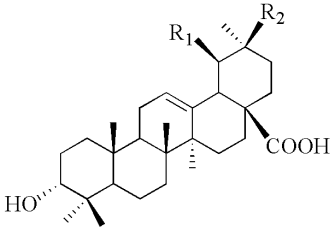
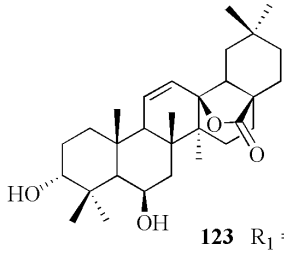
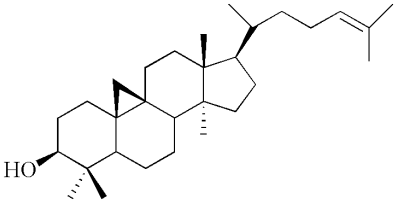


124 R_1 = H, R_2 = Me
125 R_1 = Me, R_2 = H



123 R_1 = R_2 = H



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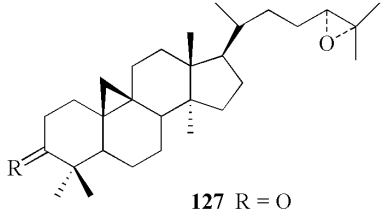


127 R = O
128 R = b-OH, a-H

Fig. 4 continued. Terpenoids.

with antimycobacterial activities against MTB H37Ra with MIC values ranging from 12.5 to 100 μg/mL (Mongkolvisut and Sutthivaiyakit, 2007). All the compounds except **100** also demonstrate antimalarial activities The clerodane diterpenes **104-109** isolated from the bark of *Casearia grewiifolia* a plant widely distributed in Thailand exhibited antimycobacterial activity against MTB H_{37}Ra. The MIC values are 12.5 μg/mL for all the compounds except **108** which has a MIC value of 25 μg/mL. The compounds also are cytotoxic towards some cancer cell lines (Kanokmedhakul et al., 2005a). The diterpenoid (*E*)-phytol **110**, is one of the antimycobacterial constituents isolated from *Morinda citrifolia* (Saludes et al., 2002) and the main antimycobacterial constituent of the Kenyan Shrub *Leucas volkensii* (Rajab et al., 1998). It is active against MTB H_{37}Rv with a MIC value of 0.95-3.8 μg/mL. The nor-diterpenoid 12-demethylmulticauline **111** has a MIC of 0.46 μg/mL comparable with rifampin and even less than that for ethambutol (Cantrell et al., 2001). This compound was the most potent against the H_{37}Rv strain of MTB of the three other aromatic norditerpenoids and two abietane diterpenoids earlier isolated from the roots of *Salvia multicaulis* (Ulubelen et al., 1997). A good number of phobol esters have also been reported to show moderate to good antimycobacterial activities. One of the phobol esters **112** from the tree *Sapium indicum* (Euphorbiaceae), exhibits activity against MTB H_{37}Ra (MIC 3.12 μg/mL) comparable to the positive control, kanamycin sulfate, but not as active as isoniazid. The stereochemistry at position 4 is very important to the activity of this compound (Chumkaew et al., 2003). The secokauranes **113** and **114** from *Croton kongensis* (Euphorbiaceae) (Thongtan et al., 2003) show significant activity towards MTB H_{37}Ra with MIC values of 25 and 6,25 μg/mL respectively.

Hopane terpenoids, **115** from *Sapium haematospermum* (Woldemichael et al., 2004) and betulin **116**, and betulinic acid **117** isolated from *Valeriana laxiflora* (Gu et al., 2004) respectively, inhibit the growth of MTB H_{37}Rv (ATCC 27294) with MIC values of 13.4, 30, 62.1 μg/mL. Oleanane triterpenes with antimycobacterial activities were also isolated from these plants. The oleanane triterpenes **118, 119** as well as the ubiquitous oleanolic acid from *Lantana hispida*, exhibit moderate activity against MTB H_{37}Rv (MIC 50 μg/mL **118**, **119** and 25 μg/mL for oleanolic acid). These compounds were also tested against a series of drug-resistant variants of H37Rv strains with similar results. The 3β,25-epoxy oleanane triterpenoids **120** and **121** isolated and from *Lippia turbinata* and from other *Lantana* species (Barre et al., 1997; Watcher et al., 2001; Okunade et al., 2004) exhibit antimycobacterial activity against MTB H_{37}Rv with MIC values of 38, 36 μg/mL respectively (Watcher et al., 2001). Of particular importance is the 18β,28-Epoxy oleanane triterpene, Aegicerin, **122** from *Clavija procera* that was tested against 37 different sensitive and resistant strains of MTB H_{37}Rv

and showed activity with MIC ranging between 1.6 and 3.12 µg/mL (Rojas et al., 2006). The epoxy oleanane **123** isolated from the *Junella tridens* and several other oleanane and ursane triterpenes including the well known oleanolic acid **124** and ursolic acid **125** exhibit growth inhibition activities towards MTB H_{37}Rv. MIC values for **123-125** are respectively 64, 50 and 15 µg/mL (Caldwell et al., 2000; Copp, 2003). The cycloartane triterpenoids **126-128** exhibit modest activity towards MTB H_{37}Rv (MIC 12.5, 8.0, 8.0 µg/mL respectively). **127** and **128** also show weak activities towards the vero cell line (Cantrell et al., 1996; Saludes et al., 2002).

5. Steroids and saponins

The plant *Morinda citrifolia* (Rubiaceae), afforded among others sterols that are strongly active against MTB H_{37}Rv with MIC values ranging from < 2 to 32 µg/mL (Okunade et al., 2004). *Thalia multiflora*, an aquatic plant from South America afforded nine sterols and other classes of compounds. Four of the sterols **129-132** show strong activities (MIC 1.0-4.0 µg/mL) against MTB H_{37}Rv (ATCC27294) (Gutierrez-Lugo et al., 2005). The naturally

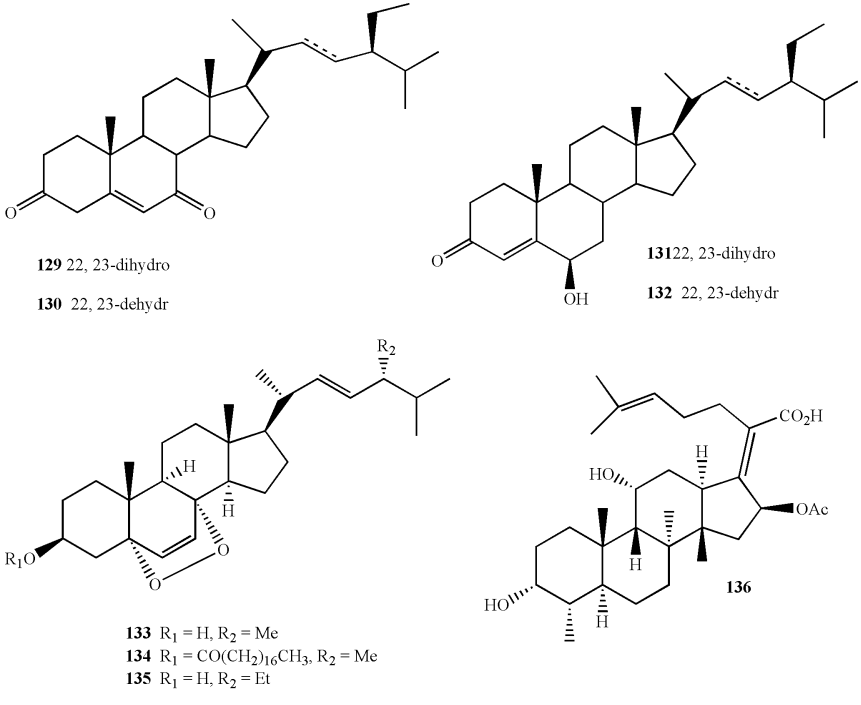
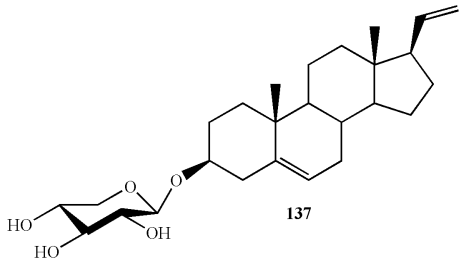
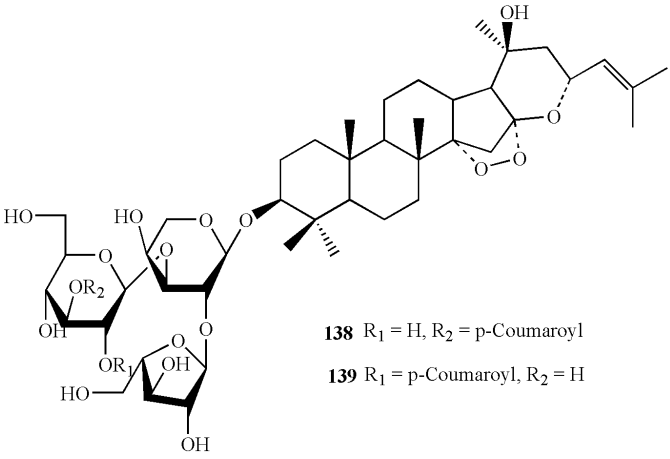


Fig. 5. Steriods/saponins.





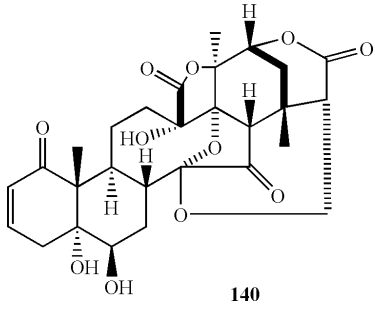


Fig. 5 continued. Steriods/saponins.

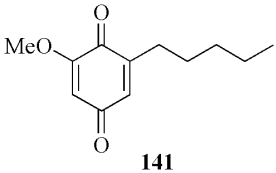
occurring ergosterol peroxide **133** isolated from *Ajuga remota* (Cantrell et al., 1999b) and *Euphorbia lagascae* (Duarte et al., 2007) was shown to possess antimycobacterial activity. **133** and other peroxides **134, 135** from the Argentinian plant *Ruprechtia triflora* (Woldemichael et al., 2003) were also reported to have activity against MTB $H_{37}Rv$ with MIC value ranging from 2-4 μg/mL. Fusidic acid, **136**, isolated from the fungus *Fusidium coccneum* (Godtfredsen et al., 1962) is a widely used antibiotic that has been shown

to inhibit the growth of 30 clinical isolates of MTB (MIC 4, and 32-64 μg/mL) and *M. kansasii* (MIC 2-64 μg/mL) (Hoffner et al., 1990; Cantrell et al., 1996; Witzig and Frazblau, 1993). Rugutt and Rugutt (2002) studied the relationships between molecular properties and antimycobacterial activities of nine steroids including fusidic acid. They suggested that the maximum antimycobacterial activity seemed to depend on hydrophobicity and type of substituents on the phytyl moiety on the steroidal backbone. The saponin **137** (Figure 5) obtained from the gorgonian octocoral *Eunicea pinta*, (Shi et al., 2002) the jujubogenin saponin **138,** as well as **139** from the stems of *Colubrina retusa* (Rhamnaceae) (ElSohyl et al., 1999), also exhibited good antimycobacterial activity. Compound **137** was active against MTB H_{37}Rv (MIC values 6.25 μg/mL) while **138** and **139** were active against *M. intracellulare* with MIC values of 50 and 10 μg/mL respectively. The seco-steroids phasalin B, **140** from *Physalis angulata* (Solanaceae) (Januario et al., 2002) show moderate activity against MTB H_{37}Rv (MIC 32 μg/mL). Woldemichael et al. (2003) also reported some sterols from *Ruprechtia triflora* (Polygonaceae) with moderate to very good antimycobacterial activity against MTB.

6. Quinones, phenols and polyphenols

The simple quinone Primin (2-methoxy-6-pentylcyclohexa-2,5-diene-1,4-dione) **141**, a natural compound occuring in *Primula obconica* and some other plant species is a sensitizer and the source of allergic contact dermatitis, primrose dermatitis in some humans. This compound has broad spectrum antiparasitic activities and moderate activity against MTB H_{37}Rv (ATTC27294) (MIC 60 μg/mL; Tasdemir et al., 2006). Plumbagin **142** from *Plumbago zeylanica*, 3-methoxjuglone **143** from *Engelhardia roxburghiana* and lapachol **144** commonly found in most *Tabebuia* (Biginoniaceae) family all exhibit antimycobacterial activities (Mossa et al., 2004; Tran et al., 2004). A synthetically derived plumbagin **142** was shown to be active against *M. avium* and *M. smegmatis* (MIC value in each case is 12.5 μg/mL). **143** was active against MTB H_{37}Rv with an MIC value of 0.2 μg/mL while lapachol **144** exhibited activity towards *M. smegmatis* (MIC 50 mg/mL) Anthraquinones **145, 146** isolated from *Prismatomeris fragrans* (Kanokmedhakul et al., 2005b) exhibit modest activity against MTB H37Ra (MIC 25 and 50 μg/mL respectively). A bisnaphthoquinonoid, diospyrin **147**, isolated from *Euclea natalensis* showed a modest activity against drug-susceptible and -resistant strains of MTB H_{37}Rv (MIC 100 μg/mL). The synthetic aminoacetate derivative **147b** of diospyrin dimethyl ether showed improved activities (MIC 10-50 μg/mL) (Lall et al., 2003).

The isolated deoxy preussomerins palmarumycin **148** believed to be isolated from fungi associated on dried fruits of *Diospyros ehretioides* as



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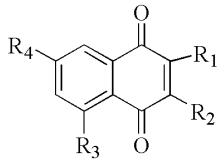
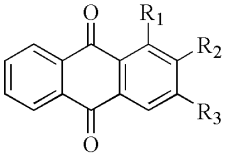
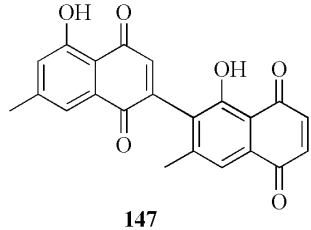


142 R_1 = Me, R_2 = R_4 = H, R_3 = OH
143 R_1 = R_4 = H, R_2 = OMe, R_3 = OH
144 R_1= OH, R_2 = $CH_2CH{=}C(CH_3)_2$, R_3 = R_4 = H



145 R_1 = OMe, R_2 = CHO, R_3 = OH
146 R_1 = OH, R_2 = CH_2OH, R_3 = OMe



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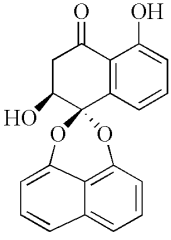

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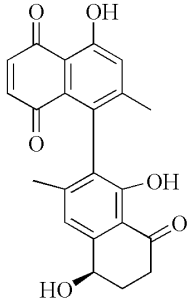

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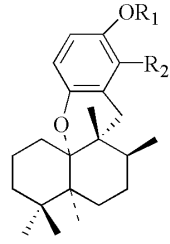
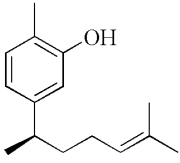


150 R_1 = $(CH_3)_2NC(S)$, R_2 = H
151 R_1 = R_2 = H



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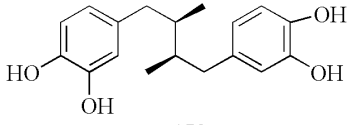

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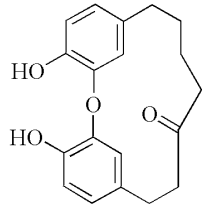
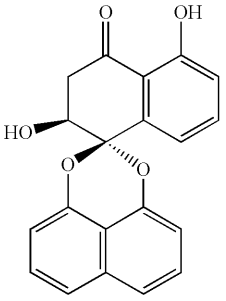


154

Fig. 6. Quinones, phenols/polyphenols.



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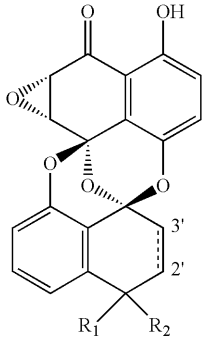
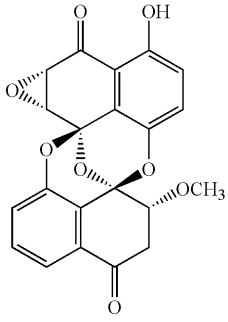


156 R_1 = a-OH, R_2 = H, 2',3'-dihydro
157 R_1, R_2 = O, 2',3'-dihydro
158 R_1, R_2 = O, 2',3'-dehydro



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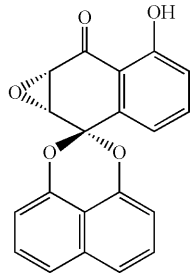

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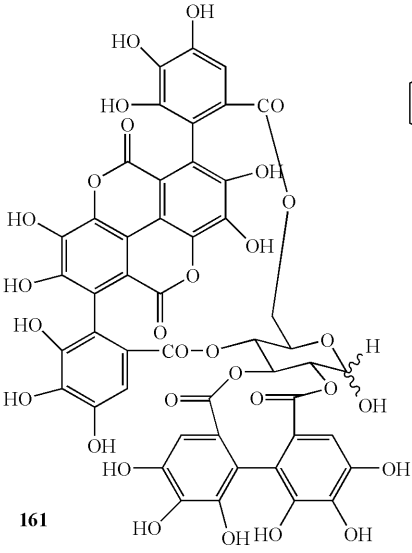


161

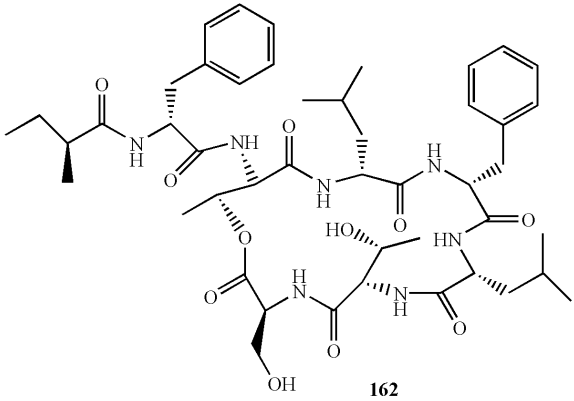
Fig. 6 continued. Quinones, phenols/polyphenols.

well as napthaquinone palmarumycin JC2 **149** from this plant exhibited activity towards MTB H37Ra (MIC, **148**, 1.56-3.12 µg/mL; **149**, 6.25 µg/mL) (Prajoubklanga et al., 2005). The thiocarbamate **150** analog of the naturally occurring sesquiterpene-phenol aureols **151** from the Jamaican Sponge *Smenospongia aurea*, was active against *Mycobacterium tuberculosis* ($H_{37}Rv$). **150** completely inhibited the growth at a concentration of 6.25 µg/mL while the natural compound **151** showed only 31% inhibition at the

same concentration (Hu et al., 2002). Phenol **152** from *Iostephane heterophylla* and **153** from *Larrea divaricata* exhibited moderate activity against MTB (Rivero-Cruz et al., 2005) MIC 16 and 50 µg/mL respectively). The tricyclic daiphenol ether engelhardione **154** has a potent activity against MTB $H_{37}Rv$ with an MIC of 0.2 µg/mL (Lin et al., 2005). The lichenous fungal metabolites preussomerins **155-160** from *Microsphaeropsis* sp were also reported to have antimycobacterial activity against MTB $H_{37}Ra$ with MIC values ranging from 3.12 to 25 µg/mL (Seephonkai et al., 2002; Prajoubklang et al., 2005,). The antimycobacterial activity of punicalagin **161** a polyphenol from *Combretum molle* (Combretaceae) was evaluated using two strains of MTB, typus humanus and an unidentified patient strain**.** It is the only tannin so far associated with antimycobacterial activity however the activity is very weak with MIC value greater than 200 µg/mL (Asres et al., 2001).

7. Peptides

Most of the peptides reported to possess antimycobacterial activities are obtained from either marine plant/organisms, fungi or bacterial sources. This is well covered in the reviews by Copp (2003), Okunade et al. (2004), Copp and Pearce (2007) and Kahalalide A **162** was isolated by Hamann et al. (1996) in Hawaii from the marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. It is the simplest of a family of cyclic peptides identified from this extract, and showed activity against *Mycobacterium tuberculosis*, inhibiting 83% of bacterial growth at 12.5 µg/mL (El Sayed, 2000). Kahalalide A does not contain obviously reactive functional groups and it is not cytotoxic to various tumor cell lines, suggesting a selective antibacterial target. This compound has recently been synthesized through a solid phase combinatorial chemistry (Bourel-Bonnet et al., 2005). Viomycin (tuberactinomycin B) **163**, a well-studied member of the family of basic cyclic peptide was once prescribed for the treatment of tuberculosis, and has been shown to block translocation during protein biosynthesis. It has a MIC value of 0.32 µg/mL when tested against *M. bovis* BCG ATTC 27291 (Oliva et al., 1998). The gene cluster encoding viomycin biosynthesis was identified and cloned from *Streptomyces vinaceus* (Yin et al., 2003). The cyclodepsipeptides enniatins **164-166** from fungi and marine sources have been reported to demonstrate modest to high antimycobacterial activity against MTB with MIC values ranging from 3.12 to 6.25 µg/mL. (Nilanonta et al., 2003) and are mostly not cytotoxic. Syringomycin **167**, a highly functionalized cyclic peptide shows a high antimycobacterial activity against a related model *M. smegmati*s with MIC of 1.50 µg/mL comparable or even lower than those obtained for the primary drugs and secondary drugs. The antibacterial thiazole peptides **168-170** isolated from the



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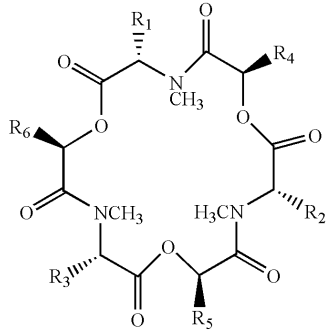
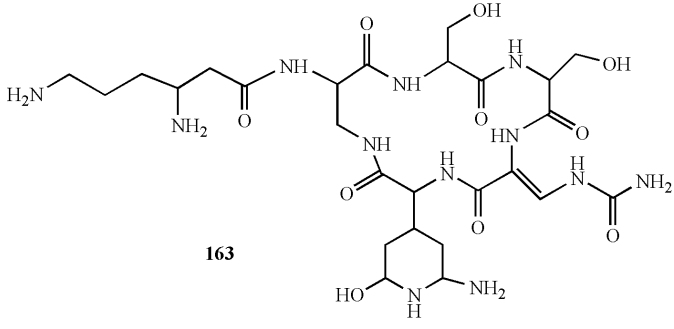
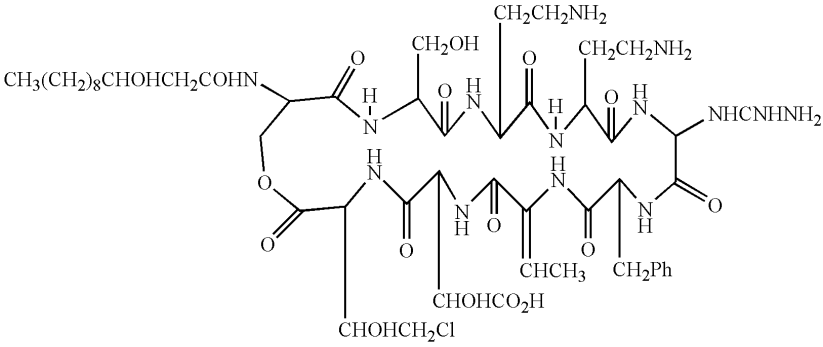


	R_1	R_2	R_3	R_4	R_5	R_6
164	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
165	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
166	i-Pr	i-Pr	i-Pr	s-Bu	s-Bu	s-Bu



163

Fig. 7. Peptides.



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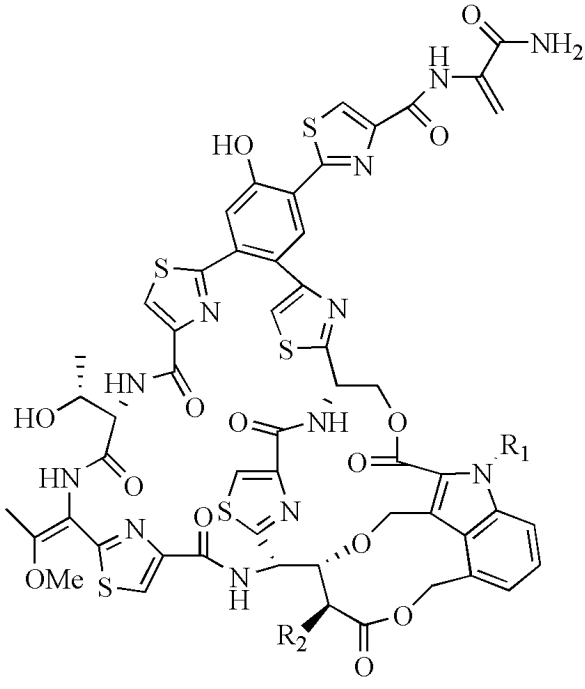
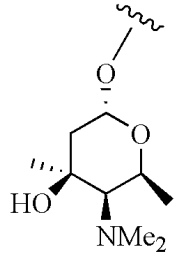




aminosugar

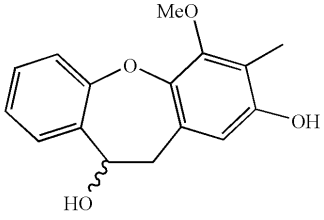
168 R1 = OH, R2 = aminosugar
169 R1 = H, R2 = aminosugar
170 R1 = R2 = OH

Fig. 7 continued. Peptides.

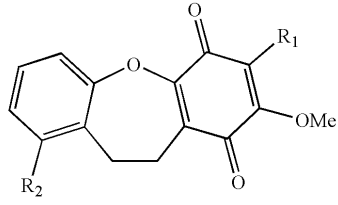
saprophytic *Nocardia* sp. (ATCC 202099) fermentation broth extraction exhibit very potent activity against MTB, *M. avium* and a variety of gram-positive pathogens (Pucci et al., 2004). Nocathiacin 1 **168** exhibits activity against MTB (ATCC 35828, MIC $\leq$ 0.008 µg/mL), *M.avium* (A26778, MIC 0.06 µg/mL) and *M. avium* (A26640, MIC 0.25 µg/mL).

8. Miscellaneous

Boonphong et al. (2007) recently isolated a number of dihydrobenzoxepins typified by compounds **171-173** and other compounds from *Bauhinia purpurea* possessing antimycobacterial activities in the microplate Alamar Blue assay. The dihydrobenzoxepin **172** has the highest activity with MIC of 24 µM against MTB $H_{37}Ra$. However, all the compounds are cytotoxic

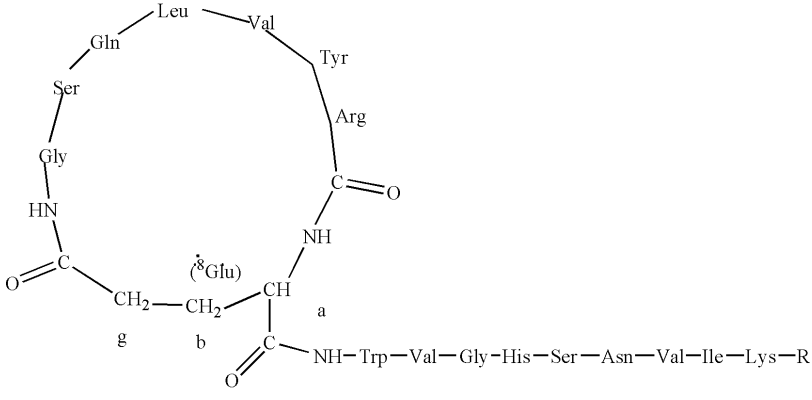


171



172 $R_1 = R_2 = H$

173 R_1 = Me, R_2 = OH



174 R = H

175 R= Gly —Pro—Pro

Fig. 8. Miscellanous.

against KB and BC cell lines. Lariatins A **174**, and B **175** inhibit the growth of *M. smegmatis* with respective MIC values of 3.13 and 6.25 μg/mL in agar dilution method, only lariatin A inhibits the growth of MTB H_{37}Rv in liquid microdilution method. Lariatins A and B were separated and purified from the culture broth of *Rhodococcus jostii* KO1B0171, which was isolated from soil aggregates collected in Yunnan, China (Iwatsuki et al., 2007).

Conclusion

The goal of identifying suitable new TB drugs is multifaceted and the current focus in drug discovery efforts is to identify compounds which are bactericidal, are mechanistically different from those currently used so that cross-resistance does not develop, and are effective against non-replicating, persistent types so as to shorten the duration of therapy. They should also be suitable for oral daily dosing with few toxic side reactions, and be cost effective for global applications. (Goldman, 2007; Personal communication).

While broad screening efforts have identified a multitude of bioreactive and structurally unique compounds, most have been published before even preliminary toxicity testing has taken place. Furthermore when they have been subject to additional testing for their toxicity, bioavailability and potency profiles in animal models their promise as possible new TB drugs has been disappointing. These results are not unique to this endeavor and have been commonplace in the pharmaceutical industry whenever this approach has been undertaken in the past.

Under-appreciated for their potential are those unique compounds which have been derived from ethnomedical leads. This is particularly true of plants used as traditional medicines for the treatment of respiratory infections and even tuberculosis. Noteworthy among these would be those remedies, which are ingested and possibly low in toxicity, rather than inhaled or utilized in other ways e.g., rubifacient. Too frequently their bioreactive structures have been published before adequate testing has shown their parameters of dosage, safety and efficacy, particularly for drug resistant and recalcitrant types of infections. This has resulted in many promising candidates being lost to drug discovery programs of the pharmaceutical industry who would mandate that patent protection is a necessary prerequisite to development.

To overcome this issue with certain viable candidates already in the public domain, semi-synthesis of known bioreactive molecules might be a way of rescuing the potential of these candidates. Moreover, these strategies may not only reduce their toxicity, but also improve their potency and bioavailability. In this regard, a good number of phytochemicals have the potential as lead compounds but so far only a few have been studied

beyond preliminary functional testing. While inexpensive preliminary toxicity studies *in vitro* have their limitations, they nonetheless are useful in providing needed information as to whether or not additional costly preclinical animal trials are warranted. Furthermore there is always the risk that promising results elicited in preclinical studies may not be applicable to humans (Goldman, 2007; Personal Communication).

The carbazole alkaloids are examples of lead compounds being derived from an ethnomedical source. These have shown moderate but consistent activity across the literature and are good candidates for further studies (Ma et al., 2005). Notwithstanding a few number of plant derived compounds have been tested *in vivo*. Two examples discussed below include the alkaloid tryptanthrin **36** that has been well studied because of its potency, simple structure and ease of synthesis. It has been shown that when a panel of multiply drug-resistant strains was utilized its potency was even superior to that of isoniazid. Structural-activity studies have also been carried out. One of the analogs, compound PA 005 was tested *in vivo* in infected mice (rBCGlux) with unsatisfactory results and hence was not pursued further. It was effective against rBCGlux in macrophages at 0.23 mg/L comparative to isoniazid but inferior to it when tested at 0.06 or 0.015 μg/mL. It was modestly effective when given orally at 50 mg/kg to infected mice (rBCGlux) (Mitscher and Baker, 1998). Another example is the pyranocoumarin (+)-calanolide A **78** Efficacy evaluations in macrophages revealed that the compound significantly inhibited intracellular replication of *M. tuberculosis* H_{37}Rv at concentrations below the MIC observed *in vitro*. Preliminary mechanistic studies indicated that it rapidly inhibits RNA and DNA synthesis followed by an inhibition of protein synthesis (Xu et al., 2004). Using **78** as a template, a library of compounds (over 60) has been produced and screened against MTB H_{37}Ra. Seven of these have MIC less than 12.8 μg/mL and four of them were bactericidal (Xu et al., 2006).

To be able to take more of these compounds beyond the preliminary screening there is no doubt that more research money is needed, with ready access to specialty testing laboratories being a primary consideration. It is hoped that the agencies funding this research will encourage researchers to consider needed additional research before they rush their discovery into publication.

As recommended earlier, (Elvin-Lewis, 2006), the low cost, provisional patent mechanism in the United States should provide American researchers with needed protection during the period of a year when toxicity and other mechanistic assays are being conducted. Investigators in other countries should be aware of national or international patent laws which can afford needed protection during this critical period.

Acknowledgement

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Color Plate Section

Chapter 11

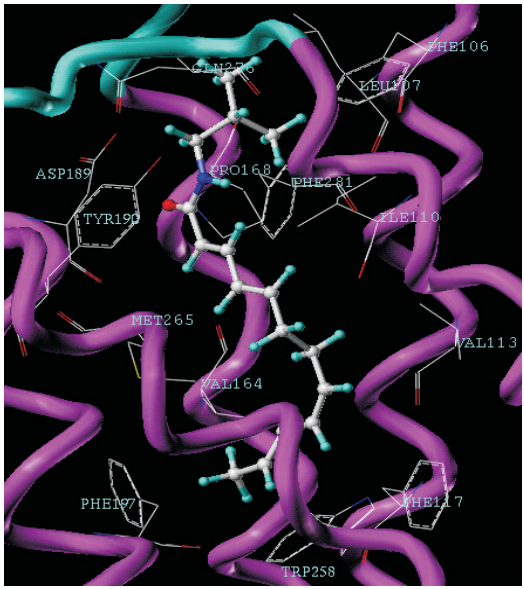

Fig. 8. Putative CB_2 receptor binding conformation of *N*-alkyl amides **22** and **23** based on a CB_2 homology model (Raduner et al., 2006).

Chapter 14

Fig. 1. TCM products are sold on traditional medicinal markets in China since many centuries. Today, they represent a valuable resource for the development of molecular targeted and individualized treatment options for cancer patients.