Functional Foods, Nutraceuticals, and Degenerative Disease Prevention

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Edited by

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Preface

In recent years, there has been a tremendous increase in the awareness of dietary habits and their influence on maintaining good health through disease prevention. Thus, there has been considerable popular demand for the health-enhancing, physiologically active components of functional foods, as consumers have become increasingly aware of the important link between diet and health, and the vital role that diet plays in combating chronic degenerative diseases such as cancer, type II diabetes, cardiovascular diseases, Alzheimer's, and autoimmune diseases. Functional foods and functional food ingredients/nutraceuticals and their influence on health have become common in scientific and popular literature. Apart from the common nutrients and vitamins that are obtained from food that form the building blocks of the human body, nutraceuticals present in many commonly consumed foods exert specific health or medical benefits, including the prevention and, in some cases, treatment of disease conditions, which enables their categorization as functional foods. Functional foods include fruits; vegetables; cereals, such as oats; oilseeds, such as soybeans; seeds and nuts; probiotics; and fish that are known to have health benefits. The nutraceuticals include bioactive ingredients in the functional foods, such as polyphenols (anthocyanins, resveratrol, various types of flavonoids, green tea polyphenols such as Epicatechin gallate, epigallocatechin gallate), carotenoids (beta-carotene, lycopene, lutein, astaxanthin), phytochemicals from herbs (rosmarinic acid), phytosterols, omega-3-fatty acids, and so on. Nutraceuticals can be derived from plant, animal, and microbial sources, including those from the aquatic environment. Phytochemicals are chemical compounds produced by plants such as alkaloids, polyphenols, and uncommon amino acids. Several of these compounds are non-nutritive in the traditional sense, but effective in preventing or combating disease. Traditional knowledge about the healing properties of foods has increasingly been substantiated with respect to their health beneficial claims, through systematic epidemiological studies and scientific investigations on the mode of action of specific functional food ingredients. Recent recommendations by the government agencies on food consumption habits take into account such studies on the disease-preventive and health- restorative roles of foods. In short, the interest in functional foods and nutraceuticals at various levels of society has resulted in an explosive growth in the literature on these topics and has enhanced the growth of a segment of the food industry worth over several billion dollars across the world, comprising health foods and drinks, sport foods and drinks, various

supplements including capsules and tablets of purified nutraceuticals, and cosmeceutical preparations.

At present, a large proportion of the scientific literature on functional foods, nutraceuticals, and disease prevention lies buried in the literature. The objective of this book is to bring some of this key information together. In the context of the growing incidence of chronic degenerative diseases across the world and associated increases in healthcare costs and loss in productivity, information focused on disease prevention will be of great interest.

This book is a compilation of different aspects of functional foods and nutraceuticals, focusing on their mechanisms of action in the human body leading to disease prevention in terms of their efficacy and highlighting the biochemical and molecular mechanism of action of their ingredients. The objective is to enhance the knowledge and understanding of functional food and nutraceuticals so that readers at various levels will be able to appreciate and use the information compiled in this book to adopt healthy lifestyles and advance health education. The discussion of the biological effects of a variety of functional foods will provide a wholesome approach to the maintenance of health through judicious choice of functional foods. The book is primarily intended for an audience comprising researchers, industry professionals, food scientists, medical professionals, and students.

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About the Editors

Gopinadhan Paliyath is a Professor at the Department of Plant Agriculture, University of Guelph, Ontario, Canada. Dr. Paliyath obtained his B.Sc.Ed. degree (botany and chemistry) in Science Education from the University of Mysore, M.Sc. degree (botany) from the University of Calicut, and Ph.D. degree (biochemistry) from the Indian Institute of Science, Bangalore. He did postdoctoral work at Washington State University, the University of Waterloo, and the University of Guelph. The major focus of Dr. Paliyath's work is to understand the mechanism of action of nutraceutical components in fruits and vegetables in humans and their implications in the prevention of chronic degenerative diseases. Dr. Paliyath also serves as the Research Programme Director of "food for health," a major theme of research under the University of Guelph/Ontario Ministry of Agriculture, Food and Rural Affairs partnership.

Marica Bakovic is a Professor at the Department of Human Health and Nutritional Science, University of Guelph, Ontario, Canada. Dr. Bakovic obtained her B.Sc. degree in chemistry from the University of Belgrade, M.Sc. degree in food science and nutrition from the University of Belgrade, and Ph.D. degree in biological chemistry from the University of Alberta. She did postdoctoral work at the University of Alberta before joining Guelph. Dr. Bakovic's research is focused on nutrigenomics, specifically in relation to phospholipid metabolism, choline transport, obesity, and metabolic syndrome in general. One of Dr. Bakovic's current research projects are on developing strategies for obesity reduction through vegetable consumption.

Kalidas Shetty is a Professor at the Department of Food Science, University of Massachusetts. He received his B.Sc. degree from the University of Agricultural Sciences, Bangalore, India, majoring in applied microbiology. He received his M.S. and Ph.D. degrees from the University of Idaho, Moscow, specializing in microbiology. He pursued postdoctoral research in plant biotechnology at the National Institute of Agro-biological Sciences, Tsukuba Science City, Japan, and Horticultural Science, University of Guelph, before joining the University of Massachusetts. Dr. Shetty has conducted extensive research on functional foods, including functional food ingredients, the influence of processing and storage in the content of bioactive ingredients, and their mechanisms of action in the human body. He has conducted extensive research on herbs and their disease-preventive potential

by altering the mitochondrial metabolism. Dr. Shetty has served as Jefferson Science Fellow at the U.S. State Department, advising the Bureau of Economic and Business Affairs on scientific issues as they relate to international diplomacy and international development. He has received the Asia-Pacific Clinical Nutrition Society Award for his outstanding contributions in the area of functional foods and human health.

1 Functional Foods, Nutraceuticals, and Disease Prevention: A Window to the Future of Health Promotion

Gopinadhan Paliyath and Kalidas Shetty

1.1 CHRONIC DEGENERATIVE DISEASES IN MODERN SOCIETY: IMPLICATIONS ON LIFE QUALITY, PRODUCTIVITY, ECONOMIC BURDEN

The diet of early humans was quite varied and reflected an omnivorous food habit. Being hunter-gatherers, humans used the fruits, vegetables, and tubers that grew wild. It has been proposed that Paleolithic humans used fruits and vegetables as a major source of food (Eaton et al., 1997). A transition from food gathering into organized agriculture may have further diversified the diet. Recognition of the healing properties of foods and plants may have been discovered accidentally; it has been transferred from generation to generation. In the literature of many ancient cultures, there is recognition of the healthy aspects of fruit and vegetable consumption. The ancient texts of the Ayurveda refer to the medicinal and healing properties of several fruits, vegetables, herbs, and spices, which were also used for food purposes. Fruits such as apples, dates, pomegranates, and grapes, prevalent in the Mediterranean, were frequently mentioned in the Biblical literature. These fruits were associated with eternal life in ancient Egyptian and Sumerian culture.

1.1.1 Diet and lifestyle changes: the missing foods

We have observed tremendous changes in the food habits of humans in the past few hundred years; changes that have been even more dramatic in the post-World War II era. The search for a sea route to the spice-growing areas of the East and the subsequent discovery of continents and new forms of edible fruits and vegetables not only enhanced the food variety but also led to the transoceanic migration of several food crops (e.g., the introduction of tomato and potato to Europe and other parts of the world where these were traditionally not grown). However, geopolitical diversities, modern science, and economic changes all resulted in the development of new social food habits. Continued changes in the processing technologies to meet consumer preferences and lifestyle changes have caused the present state of increased caloric intake, sedentary habits, overconsumption of high energy foods

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due to increased portion sizes, and low intake of functional foods, resulting in a significant increase in the prevalence of several chronic degenerative diseases, such as type 2 diabetes, cardiovascular diseases, neurodegenerative diseases, and inflammatory diseases.

1.1.2 Social and economic burden of chronic degenerative diseases

"Metabolic syndrome" refers to an aggregate of several physiological conditions that are indicators of the potential for future development of type 2 diabetes, cardiovascular diseases, and associated health problems. In general, obesity is an initial indication of the development of several other abnormalities such as hypertension and insulin resistance, further magnifying the complications. Thus, coronary heart disease (CHD), obesity, and type 2 diabetes have reached epidemic proportions around the world, and are the leading causes of the loss of living quality and productivity, as well as the high mortality rate in developed and developing countries (Bisbal et al., 2010). Recent estimations indicate that cardiovascular diseases, diabetes, obesity, cancer, and respiratory conditions account for 59% of the 56.5 million deaths annually (Jaganath, 2008).

Across the world, more than 220 million people have been estimated to be affected by diabetes (WHO Fact Sheet, 2011), and among these, type 2 diabetic incidences exceed 90% of the diagnosed cases. A recent report suggests that diabetic cases in the North American and Caribbean region have reached 37.4 million (International Diabetes Federation, 2010). Additionally, type 2 diabetes has been increasing among children in epidemic proportions (Kaufman, 2002). In North America, the occurrence of type 2 diabetes is more prevalent in African-American, Mexican-American, Native-American, and Asian-American children and young adults. However, the increase in obesity-linked diabetes is not limited to North America; it also occurs in affluent regions of other parts of the world, especially in economically emerging regions of Asia. Across the world, cases of type 2 diabetes are increasing rapidly, and such a rapid increase in a short period of time suggests the influence of environment, diet, and lifestyle risk factors in increases of type 2 diabetes. Genetic changes are unlikely to be predominant causative factors for a rapid increase in diabetes, but inherent genetic susceptibility can enhance the chances of its development. Type 2 diabetes is associated with obesity, and the number of people suffering from type 2 diabetes is predicted to rise to more than 350 million by 2030 (WHO/FAO, 2003).

Chronic degenerative diseases in general result in significant losses to society and the economy, in terms of lost productivity, increase in human suffering, and loss of living quality. In addition, the cumulative effects of the diseases create a heavy burden on the health-care system. The health-care costs and economic burden due to various types of cancer in the United States have been estimated to be in billions of dollars (Yabroff et al., 2007; healthservices.cancer.gov). Annual estimates of new cancer cases in the United States are estimated at over 1.5 million, with mortality close to half a million. According to the National Cancer Institute ("The Cost of Cancer"), the direct cost of cancer care was estimated at $104 billion and the indirect cost in terms of lost productivity and economic loss was estimated at $134 billion in 2006.

Diabetes is another disease whose costs weigh heavily on the health-care system. Diabetes can cause both microvascular (retinopathy, nephropathy, and neuropathy) as well as macrovascular (heart attacks, stroke, and peripheral vascular disease) complications.

Type 2 diabetes is the leading cause of blindness and end-stage renal failure in the United States (Klein, 1995). The risk of heart disease and stroke are two to four times more frequent in patients with diabetes; 50% of people with diabetes die due to cardiovascular disease. Diabetes, along with cardiovascular disease, has a significant socioeconomic impact on individuals, families, health systems, and countries. Recent estimates (Dall et al., 2010) suggest that economic burden from prediabetes and diabetes reached $218 billion in the United States in 2007, while the estimates in Canada were close to US$2 billion. The World Health Organization reported that between 2006 and 2015, China will lose US$558 billion in foregone national income due to heart disease, stroke, and diabetes alone. Type 2 diabetes and cardiovascular disease have genetic causes, but other factors such as obesity, physical activity, and food intake have been shown to influence the pathophysiology of both diseases significantly (Fitzgerald and Parekh, 2009). Thus, by adopting healthy living habits and reducing the development of chronic diseases, significant reductions in health-care costs can be achieved, with several indirect social and economic impacts.

1.2 HEALTH REGULATORY PROPERTIES OF FOODS: "PREVENTION IS BETTER THAN CURE"

Dietary patterns vary across the world, according to traditionally based, locally available food. However, increased globalization and changes in lifestyle habits have resulted in variations from traditional patterns, leading to an increased prevalence of chronic degenerative disease (Kris-Etherton et al., 2002). Daily intake of refined processed foods with a high glycemic index has been linked to increased risk of obesity, type 2 diabetes, and cardiovascular disease. Pancreatic β-cell dysfunction, dyslipidemia, and endothelial dysfunction are related to these problem diets (Törrönen et al., 2010). Abnormal glucose homeostasis can result in a multi-symptom disorder of energy homeostasis that includes obesity, hyperglycemia, impaired glucose tolerance, hypertension, and dyslipidemia (Burton-Freeman, 2010; Hanhineva et al., 2010).

Several "healthy spots" have been recognized in the world. People living in these areas have characteristic diets and consume foods that may be primarily responsible for their health and longevity. Food habits in the Mediterranean region, Okinawa island (Suzuki et al.), and other regions in Asia lead to some general associations. Overeating, in comparison with eating only what is sufficient, is a factor that tends to promote health abnormalities. Mediterranean food is rich in fiber, fish, and polyphenols (red wine), with a reduced amount of red meat intake compared with the typical North American diet. This has been associated with reduced incidences of cardiovascular diseases (the so-called French paradox). Increased longevity among Okinawan people has been associated with consumption of fish and vegetables such as bitter melon and a range of legumes. Consumption of spices such as turmeric and cumin in the Indian diet has been associated with reduced incidence of cancers. Above all, an active and stress-free life is a key factor that determines the longevity and health of one's life. The major characteristic of the Mediterranean diet to protect against diabetes includes a high intake of fiber, high intake of vegetable fat (in the form of monounsaturated fatty acids such as in olive oil), a low intake of *trans* fatty acids, and a moderate intake of alcohol (Martinez-Gonzalez et al., 2008). Details on several dietary patterns are available from several government sources.

1.2.1 Fruit and vegetable consumption and disease prevention

Fruits and vegetables are rich sources of a wide range of vital micronutrients, vitamins (provitamin A carotenoids, vitamin C, and folate), phytochemicals (non-provitamin A carotenoids and polyphenols), and fiber (Amiot and Lairon, 2010; Chapter 2). These components with a wide range of chemical structures and functionality provide different beneficial effects beyond simple nutrition, resulting in improved health. In general, fruits and vegetables are generally low-energy foods because of a high proportion of nondigestible carbohydrate polymers such as cellulose and pectin and lower levels of proteins and lipids. Thus, fruits and vegetables supplement the high-energy foods in diet and can be seen to provide a balance in the transit of food through the gastrointestinal tract (GIT), as well as to aid digestion and subsequent action by the gut microflora in the large intestine. The influence of fruit and vegetable components (prebiotics) in intestinal health, through viscosity modification of foods during their transition through the GIT, immunity modulation, prevention of inflammation, and maintenance of an ideal population of microflora (probiotics) is continuously being unraveled. Thus, secondary plant products such as carotenoids, polyphenols, sulforaphanes, indoles, and essential oils, in conjunction with a milieu of polymeric substances from fruits and vegetables, enhance the healthfulness of foods (Chapter 2). Nutritional recommendations in the majority of developed countries encourage increased consumption of fruits and vegetables (National Academy of Sciences, United States; Health Canada). Extensive campaigns to achieve this target (five-a-day or more) are reported to be successful in many areas, but consumption of fruits and vegetables is still below the recommended level in many countries. The influence of increased fruit and vegetable consumption and decreased incidences of several forms of cancer has been highlighted in several epidemiological studies (e.g., Steinmetz and Potter, 1991; Chapters 5, 6, 8, 11). An intake of fresh fruit and vegetables in an adequate quantity (400–500 g/day) is recommended to reduce the risk of cardiovascular disease, stroke, and high blood pressure (Jaganath, 2008). In the United Kingdom, for optimum health, it is advised to consume five portions of fruit and vegetables (each comprising at least 80 g) on a daily basis (Williams, 1995).

Plant-based foods have been utilized for therapeutic purposes since ancient times and are still being extensively used today. The Ayurvedic system in India has used medicinal plants, herbs, and foods for preventing the development of diseases and also as a means of healing (nccam.nih.gov/health/ayurveda/D287_BKG.pdf; www.ayurvedahealth.org/symposium09.pdf). Ayurveda has approached disease prevention and cure in a holistic manner, and when the root causes of many diseases involve abnormal functioning of the body at multiple levels, targeting these with various types of ingredients is only logical. The mechanistic aspects of the action of several components of fruits and vegetables have been revealed through several studies, and new aspects are still being discovered. A fundamental property of several functional food ingredients, specifically those from fruits and vegetables, is that they are very strong antioxidants, and are sometimes as efficient as vitamins C and E. The conjugated structures of these components can accept unpaired electrons and form a stable structure and gradually detoxify these through the enzymatic antioxidant system. Although the beneficial properties of functional food components have been joined to their antioxidant properties, this is only partly true. Apart from being strong antioxidants, these components are able to modulate biochemical pathways within the cell, especially when some of these pathways are overactive, such as in the case of cancer. Inhibition of calcium-calmodulin-mediated biochemical reactions by polyphenols and inhibition of cyclooxygenase by curcumin (active ingredient in turmeric) are examples of

specific inhibition of enzymes. Still at another level, functional food ingredients can influence gene expression. Several genes that are upregulated during inflammation and cancer development, such as tumor necrosis factor alpha, interleukins, protein kinase C, cyclins, and cyclin-dependent kinases, are downregulated by functional food ingredients. Phenolic compounds are also strong inhibitors of carbohydrate-digesting enzymes such as α-amylase and α-glucosidase and can function in hyperglycemia management linked to type 2 diabetes (Chapters 13 and 14). Therefore, natural forms of α-amylase and α-glucosidase inhibitors from plant-based foods provide dietary strategies to control postprandial hyperglycemia, and could be used in therapies with minimal side effects (Da Silva et al., 2010). Thus, several functional food components are able to provide multilevel protection to the body from abnormalities.

In addition to understanding the mechanisms behind the health beneficial properties of functional food components, there are several aspects that need to be understood. Most of the studies demonstrating health beneficial properties of these components were conducted using *in vitro* systems, and the observations were generally extended to whole body situations. In most of these studies, unusually high levels of components were tested, which appear to be unrealistic when the bioavailability of these components is taken into consideration. The absorption of polyphenols is very low, in the range of 0.1% to less than 1%, leading to plasma levels of around 1 μM or less. Therefore, even if one consumes large amounts of polyphenol-containing foods, the plasma level of the ingredients could be low. At present, it is not clear if this is a defense mechanism to prevent excessive absorption of these compounds. Considering the evolutionary significance of this mechanism, this may be protective. The interesting aspect is that even at the low levels absorbed, these components are active as antioxidants, modulate second messenger systems and biochemical pathways and, above all, modulate gene expression. In several *in vitro* studies, polyphenols were selectively able to kill cancer cells without affecting the growth and proliferation of normal cells at 1–2 μM levels, at which concentration these show antioxidant activity and modulation of biochemical pathways. Thus, it is likely that through constant consumption of adequate amounts of functional foods, a threshold concentration of functional food components could be built up within the body at a protective level. If cancer cells originate through mutation, these cells are likely to be killed at the level of functional food components present within the body through consistent consumption within a balanced diet. And this may be the protective mechanism that gets translated into disease prevention. Consumption of functional foods that can contain several grams of polyphenols does not appear to cause any problems as seen in many culture-specific food systems, and achieving a few micromolar levels of these ingredients appears achievable.

Another important aspect of consuming functional foods is that, whether all the ingredients are absorbed or not, the foods containing these ingredients are in contact with the inside cell layers of the GIT, which is a primary site of immune response and initiation of several abnormalities, including inflammation. Protecting these cells is as important as protecting the internal cells of the body. The internal cell layers of the GIT are the primary site of exposure to several food and nonfood chemicals, some of which may even be harmful. In some way, functional food components may have a protective role during the transition of foods through the GIT. In addition, once these reach the intestine, a beneficial microflora can digest and positively modulate the food matrix, releasing several bound functional food components that can get absorbed and enter into the circulation. The large intestine is thus a site with an increased potential to develop several abnormalities, and adequate levels of functional food consumption may have a role in preventing these

abnormalities (e.g., inflammation, ulcerative colitis). Future research will provide answers to these questions.

This book provides a consolidated approach to provide evidence for the importance of functional foods in the diet. At present, there is much debate regarding the relation of diet and health. Even though the importance of functional foods is highlighted in the media, the public is exposed to conflicting messages on food consumption. It is interesting to note that several multinational food companies are using functional food ingredients at increasing levels in some popular foods. It is likely that certain types of food will be classified as potential threats to health, as even legislative bodies are moving forward to deter the consumption of foods with high levels of sugar and fat among children as a measure to prevent childhood obesity. Such pressures will increase as the cost of health care increases, and are even projected to reach 50% of the government budget, leaving little for other areas of necessity. Thus, the importance of functional foods and healthy living habits will come to the forefront of a positive social change across the world.

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2 Functional Foods and Nutraceuticals

Chung-Ja C. Jackson and Gopinadhan Paliyath

Let food be thy medicine and medicine be thy food.

—Hippocrates, ca. 460–377 B.C.

2.1 INTRODUCTION

The terms "functional foods" and "functional food ingredients/nutraceuticals" are applied broadly to foods and food constituents, respectively, that provide specific health or medical benefits, including the prevention and treatment of diseases, as well as nutritional value (the term nutraceuticals is synonymously used for functional food ingredients in several countries). The practice of using certain foods or plants for the prevention or treatment of diseases in human societies must have originated far back in time, as hunters and gatherers did so long ago. Even primates (the closest living relatives of *Homo sapiens*) are known to use medicinal herbs. Nearly 2500 years ago, Hippocrates proclaimed, "*Let food be thy medicine and medicine be thy food*." Contemporary works by the ancient Indian sage Charaka (*Charaka Samhita*) is the foundation of modern day Ayurveda which relies heavily on foods and herbs with medicinal qualities to heal the malfunctioning of the body. Our ancestors did not know why those plants produced the observed effects, and the discovery of medicinal properties of specific plants and foods was probably accidental or the result of trial and error. It is conceivable that certain edible plants and other foods (but not necessarily the ones that were consumed on a regular basis) were helpful in alleviating sickness, which led to the knowledge of the medicinal properties of such foods. Repeated trial and error could have led to the categorization of specific foods for treating particular diseases or other medical conditions. The use of specific decoctions containing phytochemicals was scientifically practiced in Ayurvedic, Greek, and Oriental systems of medicine, though the precise mechanism of the observed effects of the ingredients was not completely understood.

In modern society, there has been a widespread upsurge of interest in functional foods and functional food ingredients since the mid-1980s. Thus, there has been considerable popular demand for the health-enhancing, physiologically active components of functional foods, as consumers have become increasingly aware of the important link between diet

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and health and the vital role that diet plays in combating chronic diseases such as cancer and heart diseases.

Functional foods and nutraceuticals include the whole spectrum of foods with disease-preventive and health-promoting properties, designer functional foods where the food may be enriched with a specific health beneficial component, vitamin and mineral supplements, herbs, phytochemicals, and probiotics. Nutraceuticals can be derived from plant, animal, and microbial sources, including those from aquatic environment. Phytochemicals are chemical compounds produced by plants, which include polyphenols, carotenoids, uncommon amino acids, sulfur-containing chemicals such as those found in cruciferous and Allium-type vegetables. Several of these compounds do not directly contribute to nutrition, but are effective in preventing or combating diseases. More than 900 different phytochemicals have been identified as components of different edible plants. The disease-preventing and disease-combating properties of phytochemicals have been investigated intensively, but further research is needed to enable mankind to benefit from the medicinal properties of as yet unknown plants. The identification of phytochemicals, together with clinical trials to test their efficacy in the treatment of certain diseases, is essential. It is also necessary to investigate thoroughly the characteristics and biological activity of functional foods and nutraceuticals, such as their therapeutic or disease-preventing efficacy, proper dosage, and possible/adverse effects (e.g., interaction with prescription drugs or with other functional foods and nutraceuticals). At the same time, standardized national and international regulation (e.g., by the United States Food and Drug Administration, Health Canada, and public health authorities of the European Union) is needed to provide guidelines to ensure the safety and quality of the functional food and nutraceutical products. Important areas of future research in this field include (1) identification and quantification of promising bioactive components in functional foods, (2) standardization of bioactive components, (3) clinical and population studies to assess effects of functional foods and nutraceuticals on human health, (4) development of standard methods to enhance and ensure the levels of selected phytochemicals and other biologically active compounds in raw and processed foods, (5) establishment of proper dosage and delivery systems, (6) investigation of bioavailability and metabolism of functional foods and nutraceuticals, (7) the study of technical and safety issues that have a bearing on Food and Drug Administration (FDA) regulation and health claim evaluation, (8) examination of regulatory issues, (9) research on effects of processing on the functional food and nutraceutical products, (10) the stability of the products, and (11) interaction of functional foods and nutraceuticals with prescription and nonprescription drugs and with other functional foods and nutraceuticals.

2.2 DEFINITION OF FUNCTIONAL FOODS AND NUTRACEUTICALS

There is, as yet, no universal definition of "functional food." This term was introduced in Japan in the mid-1980s and refers to processed foods containing ingredients that aid specific bodily functions in addition to being nutritious. So far, Japan is the only country that has a specific regulatory approval process for functional foods, which, if approved for public consumption, bear a seal of approval marked "Foods for Specified Health Use (FOSHU)" from the Japanese Ministry of Health and Welfare (Arai, 1996; Hasler, 1998). With the formation of the Natural Health Products Directorate in Canada (www.hc-sc.gc.ca/dhp-mps/prodnatur/index-eng.php; February 2011), an established system for the approval of natural health products is currently in place. Similar classification and approval process

that may lead to an international standardization of procedures are in effect in several countries. In the United States, the natural products are regulated as dietary supplements. In Australia, these products are categorized under complementary medicines. In the European Union, the natural health products are regulated as drugs. The proposed definitions of this emerging and expanding category of foods include (1) "foods that, by virtue of physiologically active components, provide health benefits beyond basic nutrition" (International Life Sciences Institute); (2) "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (the Institute of Medicine's Food and Nutrition Board) (IOM/NAS, 1994); and (3) "a food, either natural or formulated, which will enhance physiological performance or prevent or treat diseases and disorders" (Wildman, 2000). Some examples of functional foods and their physiological effects are listed in Table 2.1.

Table 2.1. Examples of functional foods

Functional foods	Bioactive components (known)	Health benefit or physiological effects	Other sources
Berries (especially blueberry)	Anthocyanins and anthocyanidins	• Neutralize free radicals • May reduce risk of cancer, heart diseases, and age-related diseases	Blackberry and raspberry, brightly colored vegetables, eggplant skin, red cabbage
	Catechins	• Neutralize free radicals • May reduce risk of cancer	Tea, grapes
Cantaloupe	β-Carotene	• Fights cancer	Carrots, pumpkins
Celery	Flavones: apigenin	• Neutralize free radicals • May reduce risk of cancer	Celery, parsley
Citrus fruits	Flavanones, hesperidin, silybin, xanthohumol, limonoids	• Neutralize free radicals • May reduce risk of cancer	Citrus, milk thistle, hops
Fish oils	Omega-3 fatty acids: (They are necessary for properly maintaining human and animal health).	• May play a role to reduce risk of CVD • Improve mental function (e.g., Attention Deficit Disorder and Alzheimer's disease) • Improve visual function, anti-inflammatory effect	Especially coldwater fish (e.g., tuna, salmon, trout, halibut, and mackerel) is rich in omega-3 fatty acids because they eat other fish, algae, and zooplankton, which are high in DHA
	DHA (docosahexaenoic acid)	• A major component of brain and eye tissue	Breast milk (contains DHA)
	EPA (eicosapentaenoic acid)	• Anti-inflammatory properties	Canola oil, soybean oil, flaxseed, and some nuts
	ALA (α-linolenic acid)	• Prevents blood clotting (cause of fatal heart attacks) and lowers LDL and total cholesterol	Omega-enriched eggs

Continued

Table 2.1. *Continued*

Functional foods	Bioactive components (known)	Health benefit or physiological effects	Other sources
	Conjugated linolenic acid (CLA)	• May improve body immune system • May decrease risk of certain cancers	Cheese, meats
Flaxseed	Phytoestrogens, lignans Omega-3 fatty acids	• Phytoestrogen, antioxidant properties • May reduce risk of hormone-related cancer (breast cancer) by competing with estrogen receptors in cells and against prostate and colon cancers	Flaxseeds, sesame seeds, rye, grain products, vegetables, and berries
	Soluble and insoluble fiber	• Lowers LDL, total cholesterol, and triglycerides by blocking uptake of cholesterol and increased excretion of it from body	
Ginkgo	Ginkgo flavone glycosides	• Improve memory and blood flow to brain • May help cure Alzheimer's disease	
Green tea	Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG)	• Neutralize free radicals • May reduce risk of cancer	
Grapefruit	Flavonols, quercetin, rutin	• Anticancer and antioxidant	Grapefruits, gingko, grapes, onions
Oats (FDA approved 1st food-specific health claim; FDA, 1997)	Insoluble fiber	• May reduce risk of breast or colon cancers	Wheat bran, grain, many vegetables
	Soluble fiber (β-glucan)	• Cholesterol lowering effect (Reduces risk of CVD)	Apples, many fruits and vegetables
Olive oil (*Olea europaea*, Oleaceae)	Oleic acid, palmitic acid, linoleic acid, stearic acid	• Increase HDL, anticancer, antioxidant	*O. europaea* (olive)
Onions (yellow and red)	Quercetin	• May reduce heart disease	Tea, apples, cherries, and red wine
Rosemary (*Rosmarinus officinalis,* Lamiaceae)	Rosmarinic acid α-pinene, camphor, cineole	• Antioxidant • May prevent cholesterol oxidation • Prevent cancer and Alzheimer's disease	Rosemary
Sage (*Salvia officinalis*)	Anti-acetylcholinesterase (AchE), camphor, α-caryophyllene, cineole, limonene	• Antioxidant, estrogenic, and anti-inflammatory properties • Enhance memory (potential treatment for Alzheimer's disease)	Lamiaceae members

Table 2.1. *Continued*

Functional foods	Bioactive components (known)	Health benefit or physiological effects	Other sources
Shallots	Fructo-oligosaccharides	• May improve gastrointestinal health	Shallots, onions
Soybean: *Glycine max* L. (Leguminosae)	Isoflavones: daidzein, genistein, glycitein, and their glycosides	• May protect against heart diseases and some cancer • May lower LDL and cholesterol	Soybean products, kudzu root (*Pueraria lobata* Ohwi), red clover
	Protein	• 25 g/day may reduce heart disease (health claim approved by FDA, 1999)	Soybean products, soy protein concentrate, soy protein isolate, soy-containing food
	Soyasaponins A and B	• May help control blood cholesterol, triglycerides, and blood sugar • Contain anticancer enzymes	Soybean products
	Carotenoids, α-tocopherol (common form of vitamin E), γ-tocopherol (primary source of vitamin E in diet), fatty acids (linoleic and oleic acids)	• Act as antioxidants, protecting fats, blood, and other body fluids from free radical damage	Tomato, orange, spinach
Spinach	Flavonoids (>1500 different types), lutein	• Fight against age-related macular degeneration	Leafy vegetables, corn, egg yolk
Sunflower seed: *Helianthus annuus* (Compositae)	Vitamins B_1, B_2, B_3, E, Ca, Mg, Mn, P, K, Cu, Re, Se, Zn, complex carbohydrates, fiber, omega-6 fatty acid, protein, unsaturated fatty acids	• Immune-boosting benefits • Antioxidant • Improve skin health • Regulate blood–fat levels • Aid in tissue repair (e.g., treating eczema)	
Tomato	Lycopene	• Antiprostate cancer • Antioxidant	Tomato, guava, rose hip, watermelon, pink grapefruit
Wine, grapes	Flavonoids, Resveratrol	• Antioxidant action blocks cholesterol oxidation (lowers LDL, prevents risk of CVD)	
	Saponins (on grape skin)	• Bind to and prevent the absorption of cholesterol	Olive oil, soybeans, Yucca and Quillaja
Yogurt	*Lactobacillus* sp.	• Probiotics	Yogurt

Nutraceuticals, as defined by Wildman (2000), are naturally occurring substances in foods, which have been found to be effective in the prevention or treatment of one or more diseases, or the improvement of physiological performance, thereby enhancing human health. The term functional food ingredients has also been synonymously used for nutraceuticals. Essential nutrients may also be considered as nutraceuticals required by the human body. Examples include vitamins C and E, which function as antioxidants.

Health-conscious "baby boomers" have made functional foods a leading trend in the U.S. food industry. According to the (*Nutrition Business Journal*, 2007a,b), the global nutrition sector comprising supplements (such as vitamins and minerals, herbs/botanicals and sport, homeopathic, meal and specialty supplements), natural and organic foods, natural personal care products, and functional foods, was estimated in value at US$228.3 billion in 2006. The annual rate of growth is estimated to be around 7%. The percentage of market shares for functional foods is estimated to account for US$85.0 billion or 37.2%, and for the natural health products at US$68.3 billion or 29.9%, respectively. However, other estimates suggest a slightly lower economic impact for the functional food sector. Moreover, the great potential ability of functional foods to mitigate and prevent diseases and promote health and well-being could bring about a significant reduction in overall national health-care costs. The most prominent nutraceuticals in the Western world have included plant fibers, β-carotene, omega-3 (n-3 or ω-3), polyunsaturated fatty acids, polyphenols, and so on, and the number of known nutraceuticals has been increasing progressively. Currently, the number of recognized nutraceutical substances has increased to more than 100, the more popular ones including isoflavones, tocotrienols, organosulfur compounds, conjugated linoleic acid (CLA), carotenoids, and flavonoids. Some examples of nutraceuticals and their sources are listed in Table 2.2.

2.2.1 Effects of functional foods and nutraceuticals on major chronic diseases

2.2.1.1 Cardiovascular diseases (CVDs)

Mortality from CVD in the world as a whole is about 51%, which could perhaps be lowered considerably, if we had a better understanding of the causes and processes of pathogenesis. Inflammation of the artery lining is the initiating cause for plaque formation in the arteries. Recent results of biochemical, epidemiological, and cell culture research suggest that one of the major risk factors for CVD is inadequate intake of foods containing antioxidant micronutrients such as vitamins E and C, β-carotene, and ubiquinone (coenzyme Q10). Increased intake of fruits and vegetables containing antioxidants is recommended as a protective measure against the development of cardiovascular diseases (Duthie and Brown, 1994). Moreover, consumption of fish at least twice a week can significantly reduce the risk of suffering from heart attack or stroke. Omega-3 fatty acids are thought to play a particularly important physiological role in the prevention of CVD (Simopoulos, 1997; Dewailly et al., 2001; Holub, 2002; Hu et al., 2002). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in fish oil are considered essential fatty acids, but they can be formed in the body from another omega-3 fatty acid, α-linolenic acid (ALA). ALA is found in various foods, such as flaxseed, spinach, and marine animals that eat organisms containing ALA. ALA is included as an ingredient in several foods.

Table 2.2. Examples of vitamins and functional food ingredients (nutraceuticals) by food sources

	Food sources	Effects of health benefits
Allicin	Garlic, onions, chives, leek	• Antibacterial • Reduce risk of cancer and CVD, thinning blood
Ascorbic acid	Rose hip, fruits, peppers	• Antioxidant (e.g., regenerate oxidized vitamin E) • Reduce risk of cataracts • Reduce cold symptoms (note: not recommended if one has kidney stones or hemochromatosis)
β-Carotene	Carrot, tomato, yellow squash, broccoli, citrus fruits, cantaloupe, pumpkin, sweet potatoes, tomato, paprika, green vegetables	• Antioxidant (not recommended to smokers—incidence of increased lung cancer when administered alone)
Capsaicin	Pepper fruit	• Anti-inflammatory
Catechin	Teas, berries	• Antioxidant
Cellulose (insoluble fiber)	Most of plants (cell wall), whole grain, bran, cabbage family, peas, beans apples, root vegetables	• Hold water, reduce colonic pressure, reduce transit time of digestion • Lower cholesterol level and CVD
Coenzyme Q10 (ubiquinone)	Meat, seafoods	• Antioxidant
Cyanidin	Grapes, raspberries, strawberries	• Antioxidant
Flavonoids and anthocyanins	Fruits, many plants, red wine	• Lower cholesterol levels, anti-osteoporosis, anticarcinogenic, antioxidant
Folic acid (vitamin B_9)	Citrus fruits, vegetables, tomato, grains	• Reduce blood level of homocysteine • Decrease CVD
β-Glucan	Brans of oats, barley, wheat	• Lower blood lipid
Indoles	Cabbage, broccoli, cauliflower, kale, Brussel sprouts	• Antioxidant, anticancer agents
Isoflavones (daidzein, genistein, and glycitein)	Soybeans, other legumes, red clover	• Anti-osteoporosis, anticancer • Reduce stroke, heart disease, and hot flush
Isothiocyanates	Cruciferous vegetables	• Anticancer
Lecithins	Soybeans	• Lower LDL
δ-Limonoids	Citrus fruits	• Anticancer
Lignin	Plants	• Improve vision
Lutein	Spinach, banana, egg yolk, green vegetables	• Antioxidant • Improve vision
Lycopene	Tomato and its products	• Antioxidant • Anti-prostate
Resveratrol	Grapes (skins), red wine	• Decrease LDL and increase HDL • Antioxidant
Quercetin	Red grapes, citrus fruit, onion, apple skin, berries, tea, broccoli	• Lower blood lipid • Anti-inflammatory • Antioxidant
Selenium	Grains, Brazil nuts, seeds, meat, eggs	• Antioxidant
Soy protein	Soybeans	• Lower the LDL

Continued

Table 2.2. *Continued*

	Food sources	**Effects of health benefits**
Tocopherols	Soybean oil, olive oil, corn oil, nuts, seeds, wheat germs, peppers	• Anticancer • Antioxidant • Lower blood lipid (protect cell membrane, prevent lipid oxidation) (note: high dose of it may interfere with blood clotting)
Calcium	Milk, meat	• Bone protection
Choline	Meat, fenugreek leaves, fava beans	• Possibly to prevent and treat Alzheimer's disease
Conjugated linoleic acid (CLA)	Meat, milk	• Bone protection
Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA)	Fish oil	• Anti-inflammatory • Reduce CVD
Sphingolipids	Most membrane of animal cells	• Building block components of lipid in tissues
Ubiquinone (coenzyme Q10)	Almost all organisms	• Electron carriers for the electron transport chain in mitochondria
Zinc	Meat, milk, seafood, liver, eggs	• Requires Zn as part of many prosthetic groups of over 80 enzymes
Microbials		
Lactobacillus acidophilus (NCFB 1748)	Yogurt, fermented milk	• Probiotics, gastrointestinal health
Bifidobacterium bifidum *Bifidobacterium infantis* *Bifidobacterium longum*	Yogurt, sour milk	• Probiotics, disease prevention from harmful bacteria • Produce both acetic and lactic acid which reduce intestinal pH • Restrict the production of phenols, ammonia, steroid metabolites, bacterial toxin, and vasoconstriction-causing amines
Streptococcus salivarius sp. (*Thermophilus*)	Yogurt, soured milk	• Probiotics, beneficial role in the ecosystem of the human gastrointestinal tract

2.2.1.2 Cancer

Cancer, which is responsible for the second highest mortality rate after CVD in the "developed" countries, correlates with environmental factors and dietary habits (Milner, 1994). Epidemiological and clinical observations have provided convincing evidence that a good diet can significantly lower the risk of developing cancer. Many phytochemicals and other substances in foods appear to afford protection against cancer (Delaquis and Mazza, 1998; Oomah and Mazza, 1998; Bruno and Wildman, 2000; DiSilvestro, 2000; Guthrie and Kurowska, 2000). These include fibers, vitamins A, D, and C, vitamins of the B complex, organosulfur compounds found in *Allium* plants (i.e., garlic and onions), ellagic acid, and other phenols, flavonoids found in fruits and vegetables, and glucosinolates in cruciferous

vegetables such as broccoli (components which, on hydrolysis, yield the biologically active derivatives indole-3-carbinol and isothiocyanates such as sulphoraphane) (Jeffery and Jarrell, 2000). These multifarious essential and nonessential nutrients apparently modify the carcinogenic process at specific sites, interfering with carcinogenesis. Unhealthy diet is not the only factor that influences the risk of cancer development; however, adopting healthy dietary habits provides an easy and potentially effective means of lowering the risk. Progress is made in the understanding of biochemical and molecular mechanisms whereby components of the human diet affect the initiation and growth of cancer cells. As well, research should focus on the nutritional intervention to make it appropriate and effective, providing a basis for realistic recommendations regarding dietary regimes (Milner, 1994; Arnot, 1998).

2.2.1.3 Obesity and Type II diabetes

In recent years obesity and type II diabetes have been showing an alarming increase in the developed and developing countries, among people of all ages (World Health Organization, 2011). This phenomenon can be attributed to a number of causes, notably (1) the change from "traditional foods" (a well-balanced diet) to "fast foods" containing higher concentrations of undesirable ingredients (e.g., trans and saturated fatty acids, and large amount of sugar), (2) the hectic pace of modern life, which often prompts people to eat more convenient but less wholesome foods, and (3) lack of exercise and a largely sedentary lifestyle. Obesity increases the incidence of ailments such as heart disease, diabetes, and cancer. The number of people who are classified as obese (i.e., people whose body mass index exceeds 30) has increased, both in men and women, to over 30% of the population within the last 20 years in the United States (CDC, 2007). Dietary factors of potential importance for energy balance and fat distribution in humans include (1) macronutrients (e.g., carbohydrate, protein, and fat), (2) micronutrients (e.g., thiamin and Zn), and (3) non-nutrients (e.g., dietary fiber, caffeine, capsaicin, and phytoestrogens). It is important to understand the relation between (whether biochemical or physicochemical) energy intake, energy expenditure, and the deposition of fat at various anatomical sites. Furthermore, there are great opportunities for research designed to identify or develop functional foods that may help to ameliorate the growing problem of obesity in modern society (Wahlqvist, 1994).

2.2.1.4 Alzheimer's disease (AD)

Consumption of fish containing the omega-3 fatty acid DHA twice a week has been found to reduce the risk of AD by 60% (Morris et al., 2003). DHA "had the strongest protective effect against AD" compared to other omega-3 fatty acids. By contrast, EPA, another omega-3 fatty acid, appeared to have no protective effect, and ALA, which is also an omega-3 fatty acid, protected only a segment of the population with a specific genotype. In short, DHA may play an important part in the prevention of AD.

2.3 SOURCES AND BIOLOGICAL EFFECTS OF FUNCTIONAL FOODS AND NUTRACEUTICALS IN NATURE

Evidence from several epidemiological studies has shown that people consuming diets rich in vegetables and fruits had, on average, a 50% lower incidence of cancer than those

who consumed lesser amounts (Block et al., 1992; Potter, 1992; Hasler, 1998; Jeffery and Jarrell, 2000). However, a recent study (European Prospective Investigation into Cancer and Nutrition) involving over 500,000 adults has shown a less strong association between increased consumption (an additional two servings or 200 g) of fruits and vegetables and cancer development (Boffetta et al., 2010). However, as the population size increases, the genetic variability also increases, and coupled with variabilities in age, food, and other living habits, such a study is likely to provide an unclear result. It appears that the plant-based diets, besides providing basic nutrients, are sources of phytochemicals that reduce the incidence of chronic diseases such as cancer. Many classes of the biologically active phytochemicals have been identified earlier (Steinmetz and Potter, 1991a,b), and many more of them are being continuously identified and isolated. Phytochemicals in fruits and vegetables have important beneficial effects on human health, including disease prevention, the mechanisms of which are increasingly becoming clear. It must also be borne in mind, however, that health-enhancing products from plants are not necessarily safe for all individuals (see Section 2.6). In addition, the Nutrition Labeling and Education Act of 1990 (NLEA), which requires nutrition labeling for most foods and allows health-benefiting information on food labels, is helping to heighten awareness of functional foods and optimize their benefits (ADA, 1995; Howard and Kritchevsky, 1997). Examples of functional foods and nutraceuticals and their respective health benefits are listed in Tables 2.1 and 2.2, respectively, and selected ones are discussed in more detail below.

2.3.1 Flaxseed (*Linum usitatissimum*)

Among the major seed oils, flaxseed oil contains the highest level of the omega-3 fatty acid ALA (57%) (Oomah and Mazza, 1998), but research on flaxseed has mainly focused on lignans, a class of phytoestrogens. Flaxseed contains many important functional components, including high concentrations of protein and dietary fiber, lignin, and other phytochemicals with antioxidant activities, such as flavonoids, phenolic acids, and tocopherols (Oomah and Mazza, 1998). The two primary mammalian lignans, enterodiol and enterolactone, are formed in the intestinal tract by bacterial action on plant lignan precursors (Setchell et al., 1987; Setchell, 1995). Flaxseed is the richest source of mammalian lignan precursors (Thompson et al., 1991). Owing to the structural similarity of enterodiol and enterolactone to naturally occurring and synthetic estrogens, some scientists have claimed that these compounds exert both weak estrogenic and antiestrogenic effects. However, the affinity of the plant-derived estrogen-like components to the estrogen receptor is several-fold lower than human estrogen, and therefore these components may possess alternate mode of activities under the concentrations that are achievable in the body. In experiments on rodents, flaxseed has been shown to decrease tumors of the colon and mammary gland (Thompson, 1995) and lungs (Yan et al., 1998). Only a few studies have been conducted to evaluate the possible cancer-reducing potential of flaxseed consumption among humans. Ingestion of 10 g of flaxseed per day may cause several hormonal changes associated with reduced breast cancer risk (Phillips et al., 1993). Aldercreutz (1995) found that the urinary lignan excretion was significantly lower in postmenopausal breast cancer patients than in control subjects consuming a normal mixed or lacto-vegetarian diet. Flaxseed is also known to reduce the total and low-density lipoprotein (LDL) cholesterol levels as well as platelet aggregation (Bierenbaum et al., 1993; Cunnane et al., 1993). Flaxseed, however, cannot be digested unless it is powdered. When it is powdered and consumed, ALA is released, and

this essential fatty acid may help to prevent blood clotting, which can cause fatal heart attacks. Since the whole flaxseed contains both soluble and insoluble fiber, it can reduce constipation and diseases of the diverticula.

2.3.2 Phytoestrogens

Phytoestrogenic compounds (e.g., isoflavones, coumestans, and lignans), which are weak estrogens found in plant food such as soybean and its products, may have antiestrogenic effects. Some phytoestrogenic compounds, at the levels consumed in the typical American diet, impart a reduced risk of developing endometrial cancer. A recent investigation revealed an inverse correlation between dietary intake of food containing three classes of phytoestrogens (isoflavones, coumestans, and lignans) and the risk of endometrial cancer development (Horn-Ross et al., 2003). The relationships were slightly stronger in postmenopausal women than in premenopausal women.

2.3.3 Tomatoes

Tomatoes have been in the spotlight since the mid-1990s because of the presence of lycopene, a primary carotenoid, with strong antioxidant and health regulatory function. Consumption of products containing lycopene has been linked to a reduced risk of cancer development (Giovannucci et al., 1995; Goodman et al., 1998; Krinsky, 1998; Gester, 1999; Bruno and Wildman, 2000), especially cancers of the prostate, breast, digestive tract, cervix, bladder, and skin, and possibly the lung (Clinton, 1998). A study of more than 47,000 men revealed that those who consumed tomato products 10 or more times per week had less than half the risk of developing advanced prostate cancer than the controls (Giovannucci et al., 1995). The mechanism for prevention of cancer by lycopene may be related to the compound's role as an antioxidant and as a modulator of gene expression (see Chapter 8). Lycopene is known to be the most efficient quencher of singlet oxygen in biological systems (Di Mascio et al., 1989). This antioxidant function of lycopene may also explain the results of a multicenter European study which demonstrated that levels of carotenoids in adipose tissue were inversely related to the risk of myocardial infarction (Kohlmeier et al., 1997).

2.3.4 Garlic (*Allium sativum*)

Garlic is one of the most widely used traditional culinary herbs in the world, and it has long been noted for its medicinal virtues, including antibacterial activity. Several medicinal functions of garlic have been documented in the literature (Alder and Holub, 1997; Lawson, 1998; Nagpurkar et al., 2000). These include antihypertensive, cholesterol-lowering, cancer-chemopreventive, and antibiotic effects (Srivastava et al., 1982). The characteristic aroma and taste of garlic are due to an abundance of oil- and water-soluble organosulfur compounds (e.g., allicin), which are probably responsible for the various medicinal effects of garlic. The intact garlic bulb contains an amino acid called alliin, which is converted to allicin by an enzyme called allinase when garlic bulbs are cut or crushed (Block et al., 1992; Koch and Lawson, 1996). The allicin spontaneously decomposes to numerous sulfur-containing compounds, some of which have been shown to inhibit tumorigenesis and to reduce cancer risk in humans (You et al., 1988; Dorant et al., 1993). Indeed, they apparently

reduce incidence of colon cancer by 50% in postmenopausal women (Steinmetz et al., 1994). *Allium* vegetables in general, including onions, are believed to offer protection against cancer of the gastrointestinal tract. However, not all epidemiological studies have shown garlic to be effective against carcinogensis (Steinmetz and Potter, 1991a,b). This contradiction may reflect differences in the types and quantities of organosulfur compounds present in fresh garlic and garlic products (Lawson et al., 1991).

Garlic has also been shown to reduce the incidence of CVD and hypertension and to lower cholesterol levels (Warshafsky et al., 1993, Silagy and Neil, 1994a,b; Ernst, 1997; Isaacsohn et al., 1998). The components that inhibit cholesterol biosynthesis include *S*-allyl cysteine, *S*-ethyl cysteine, and *S*-propyl cysteine (Yeh and Liu, 2001).

2.3.5 Cruciferous vegetables

Cruciferous vegetables include broccoli, cabbage, cauliflower, Brussels sprouts, collard, kale, kohlrabi, turnip, mustards, horseradish, and watercress. The consumption of cruciferous vegetables has been shown to reduce the risk of developing several kinds of cancer (Verhoeven et al., 1996). The cruciferous vegetables have high concentrations of glucosinolates (a group of glycosides), which have been linked to the anticarcinogenic properties of cruciferous vegetables (Verhoeven et al., 1997; Delaquis and Mazza, 1998). All cruciferous vegetables store glucosinolates in their vacuoles. They are hydrolyzed by an enzyme myrosinase, which is found in the plant cells, to a variety of products such as isothiocyanates and indoles. Although natural and synthetic isothiocyanates as a whole have been shown to prevent cancer in animals (Hecht, 1995), attention is currently being directed mainly to a particular class of isothiocynates known as sulforaphanes which are isolated from broccoli. Sulforaphanes induce enzymes (e.g., quinone reductases) involved in carcinogen detoxification in the human body (Fahey et al., 1997). Three-day-old broccoli sprouts contain concentrations of glucoraphanin (the glucosinolate of sulforaphane) that are 10–100 times greater than those of mature plants (Nestle, 1998), and hence may be more beneficial. The mechanisms of cancer prevention by cruciferous vegetables are discussed in detail in Chapter 6.

2.3.6 Citrus fruits

Citrus fruits include oranges (*Citrus sinensis*), tangerines (*Citrus reticulata*), lemons (*Citrus limon*), and grapefruit (*Citrus paradisi*). There are many physiologically active components in citrus fruits, among them citrus flavonoids and limonoids, which comprise two major classes of compounds that have been extensively investigated, and essential oils containing monoterpenes. A large number of components in citrus products are pharmacologically active, and they have been shown to be capable of preventing diseases and promoting health (e.g., because of anticancer effects) (Tillotson et al., 1993; Middleton and Kandaswami, 1994; Rouseff and Nagy, 1994; Girard and Mazza, 1998). Some of the principal nutrients in citrus fruits, such as vitamins C and E, folic acid, dietary fiber, and carotenoids, are thought to play a role in preventing or delaying the onset of major degenerative diseases (e.g., cancer, cardiovascular disease, and cataracts) by counteracting oxidative processes. Several "non-nutrients," including limonoids and flavonoids, appear to block and suppress carcinogens (Hasegawa and Miyake, 1996) and may be effective against a variety of spontaneous and chemically induced rodent tumors (Crowell, 1997; Gould, 1997; Ripple et al., 1998).

2.3.7 Cranberry

Cranberry juice has been used to treat urinary tract infection, its effectiveness being attributable to the fact that it is rich in benzoic acid, which acidifies the urine (Blatherwick, 1914). In addition to benzoic acid, this juice also contains two other phytochemicals, fructose and a nondialyzable polymeric compound, which have the ability to inhibit adherence of *Escherichia coli* to uro-epitheial cells (Schmidt and Sobota, 1988). The polymer compound was eventually isolated from cranberry and blueberry juices and was found to inhibit adhesins present on the pili of the surfaces of certain pathogenic *E. coli* cells (Ofek et al., 1991). A clinical trial in which 150 subjects were allowed to consume 300 mL of juice per day for 6 months showed significant (58%) reduction in urinary tract infection compared to the control group (Avorn et al., 1994). Cranberries evidently help to combat not only urinary infection but also several forms of cancer and heart disease.

Furthermore, there is evidence that cranberries have the potential to protect animals against brain cell damage resulting from strokes, and that cranberry juice has a statistically significant tendency to diminish the occurrence of brain cell death. Stroke is the third leading cause of death in the United States and the most common cause of disability in adults (Neto, 2003). The specific phytochemicals responsible for these protective effects have not yet been characterized but may include anthocyanins, which are strong antioxidants.

2.3.8 Tea

Next to water, tea is one of the most commonly consumed beverages in the world. Tea, especially green tea, is a potent anticancer agent owing to its high concentrations of polyphenolic compounds (AHF, 1992; Dreosil et al., 1997; Harbowy and Balentine, 1997; Mueller-Klieser et al., 2002). The polyphenol content of tea leaves can amount to as much as 30% of their total dry weight. Catechins are the principal polyphenols in tea and the most significant ones from the standpoint of medicinal effects (Graham, 1992). In green tea there are four major catechins, that is, epicatechin, epicatechin-3-gallate, epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), which are flavonoid derivatives. EGC and EGCG in particular are known to be effective in preventing cancer. Although the occurrence of such effects in humans is inconclusive, it has been found that consumption of large quantities (5–6 cups per day) of green tea is associated with a decreased incidence of breast cancer in Japanese women (Kohlmeier et al., 1997; Nakashi et al., 1998). The tea polyphenols appear, moreover, to block the formation of nonmelanoma skin tumors. Unlike sunblock, which prevents the skin from absorbing harmful ultraviolet (UV) light, tea polyphenols function metabolically after the skin is exposed to excessive sunlight. The compounds found in both green and black teas inhibit a newly discovered chemical pathway involving an enzyme called c-Jun *N*-terminal kinase (JNK)-2 that appears to play a key role in the development of tumors (Dong, 2003; Maeda-Yamamoto et al., 2004). JNK-2 levels increase after the skin is exposed to sunlight, and remain elevated in the exposed area, increasing the probability of developing skin cancer. Tea may be effective only if large quantities of it are consumed, suggesting that a topical "cream" containing tea polyphenols with increased bioavailability is likely to be more effective.

Tea appears to be effective against CVD as well as cancer. A study of elderly men in the Netherlands (Hertog et al., 1993) who consumed tea as the major source of flavonoids

(e.g., quercetin, kaempferol, myricetin, apigenin, and luteolin) in their diet showed reduced mortality from CVD in this population. There are other studies demonstrating a link between consumption of tea and reduction in the incidence of CVD (Tijburg et al., 1997).

2.3.9 Wine and grapes

Increasing evidences indicate that wine, especially red wine, reduces the incidence of CVD. St. Leger et al. (1979) found a strong inverse correlation between wine intake and death from ischemic heart disease in both men and women from 18 countries. In France in particular, there is a relatively low incidence of CVD despite the prevalence of a diet rich in dairy fat (Renaud and Lorgeril, 1992). This "French paradox" has been explained, in part, by the ability of red wine to increase high-density lipoprotein (HDL) cholesterol (Das et al., 1999). The beneficial effects of red wine are thought to result from high concentrations of polyphenols, which are extracted from grape skins during fermentation and act as antioxidants. The concentrations of phenolic compounds in red grapes were found to range from 260 to 920 mg/100 g fresh weight, and red wines contained 1800 (Cabernet Sauvignon) to 3200 mg/L (Petite Sirah) of polyphenols (Kanner et al., 1994; Jacob et al., 2008). The tendency of red wine to prevent CVD could be explained by the ability of phenolic compounds to prevent the oxidation of LDL, a critical factor in the process of atherogenesis (Frankel et al., 1993a,b). Moreover, moderate consumption of red wine is associated with a decreased risk of age-related macular degeneration (Obisesan et al., 1998). Red wine is also a good source of trans-resveratrol, a stilbene phytoalexin found in grape skins (Creasy and Coffee, 1988). Resveratrol has been shown to have estrogenic (Gehm et al., 1997) and cancer preventive properties (Jang et al., 1997).

Despite the evidence for the effectiveness of wine against CVD, a study of over 100,000 adults in North California demonstrated that consumption of red wine involved no significant reduction of coronary risk (Klatsky et al., 1997). It should also be pointed out that excessive consumption of alcoholic beverages has been linked to the development of several types of cancer, such as breast cancer (Bowlin et al., 1997), as well as cirrhosis of the liver. Those who wish to enjoy the health benefits of wine without undue risk may consider alcohol-free wine, which has been shown to increase total plasma antioxidant capacity, or grape juice, which is effective in inhibiting the oxidation of LDL (Stein et al., 1999).

2.3.10 Chocolate

Chocolate contains flavonoids such as (–) epicatechin which may promote cardiovascular health as a result of direct antioxidant effects or through antithrombotic mechanisms. Dark chocolate brings about an increase in both the total antioxidant capacity and the (–) epicatechin content of blood plasma, but these effects are considerably reduced when milk chocolate is consumed, or when dark chocolate is consumed with milk (Serafini et al., 2003). This finding indicates that milk may interfere with the absorption of antioxidants from chocolate *in vivo* and may reduce the potential health benefits conferred by consumption of moderate amounts of dark chocolate (Geleijinse et al., 1999; Serafini et al., 2003).

2.3.11 Fish

Omega-3 (ω-3 or n-3) fatty acids (e.g., DHA, EPA, and ALA) comprise a class of essential polyunsaturated fatty acids obtained primarily from fish oils. DHA and EPA can be formed in the body from another omega-3 fatty acid, ALA, besides being consumed directly as fish oil. ALA is found in plant parts, such as flaxseed and spinach, as well as fish and other marine animals that eat organisms containing ALA. DHA is one of most important omega-3 fatty acids and is a major component of brain and eye tissues. The omega-3 fatty acids may play an important role in reducing the incidence of CVD, cancer, mental disorders (e.g., attention deficit disorder, bipolar disease, depression, and AD), and alcoholism (Hibbeln, 2003). In addition, omega-3 fatty acids perform many biological functions that can benefit the heart and blood vessels. They have anti-inflammatory properties, reduce the tendency of the blood to form clots, stabilize the electrical activity of the heart, lower triglyceride levels, reduce blood pressure moderately, and improve the functioning of artery linings. These findings prompted the American Heart Association to recommend the consumption of at least two servings of fish per week, especially fatty fish such as salmon, sardines, mackerel, herring, lake trout, tuna, halibut, and anchovies. Besides, the results of a study undertaken to investigate possible effects of fish consumption and the intake of DHA and other types of omega-3 fatty acids on AD indicate that weekly fish and DHA consumption reduces the risk of AD by 60% (Martek Biosciences Corp, 2003). Although fish is an important part of inuit (Eskimo) diet (Krumhout et al., 1985; Harris et al., 1990; Harris, 1997) containing high levels of fats, increased fat consumption does not increase the incidence of CVD among these people (Bang and Dyerberg, 1972).

2.3.12 Dairy products

Dairy products, such as milk, cheese, and yogurt, are among the best sources of several important vitamins (e.g., vitamin D and riboflavin) and minerals (e.g., calcium and phosphorus) (Jelen and Lutz, 1998). Calcium is an essential nutrient which prevents osteoporosis and possibly colon cancer. The fermented dairy products, such as yogurt, kefir, and sour milk are categorized as "probiotics," which are defined as "live microbial food supplements, which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1997; Jelen and Lutz, 1998; Farnworth, 2000). Probiotics have attracted considerable attention, primarily because they display anticarcinogenic, hypocholesterolemic, and antagonistic action against intestinal pathogens (Mital and Garg, 1995).

Microorganisms have been used traditionally in fermentation of milk, lactic acid bacteria being of particular interest. In studies of Masai tribesmen in Africa, hypocholesterolemic effects of fermented milk were discovered (Mann and Spoerry, 1974; Mann, 1978). The Masai have low levels of serum cholesterol and a low incidence of coronary heart disease (CHD) in spite of a high proportion of meat in their diet. It was found that they consume large quantities of fermented whole milk daily. Mital and Garg (1995) performed studies pointing to a role of probiotics in the prevention of colon cancer. It may be due to the presence of lactic acid bacteria, which alter the activity of fecal enzymes (e.g., β-glucuronidase, azoreductase, and nitroreductase) that are thought to play a role in the development of colon cancer.

2.3.13 Carbohydrates

Fermentable carbohydrates that serve as nutrient substrates for the "beneficial or good" microflora of the gut make up a class of "prebiotics" which have been defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health" (Gibson and Robertfroid, 1995; Farnworth, 2000). These include dietary fibers, nonabsorbable sugars, sugar alcohols, and fruit oligosaccharides (Tomomatsu, 1994; Gibson et al., 1996). Oligosaccharides consist of short-chain polysaccharides made up of 3–10 simple sugars linked together. They are found in many fruits and vegetables (e.g., bananas, garlic, onions, milk, honey, artichokes, and chicory). The category of prebiotics has been extended to include "symbiotics," which are mixtures of probiotics and prebiotics (Gibson and Robertfroid, 1995). Many symbiotic products are available in the European market.

2.3.14 Meat

A CLA known as an "anticarinogenic fatty acid" was first isolated from grilled beef (Ha et al., 1987). This CLA is an isomer of linoleic acid (18:2, n-6) in which the double bonds are conjugated instead of being in the typical methylene-interrupted configuration. Reportedly, there are nine different isomers of CLA that occur naturally in foods. CLAs have been shown to be effective in suppressing stomach tumors in mice and mammary carcinogenesis in rats (Ip and Scimeca, 1997). It has even been suggested that they play a role in reducing weight (Park et al., 1997).

The highest concentrations of CLAs are found in the fatty fractions of foods obtained from ruminants—for instance, beef, dairy products, and lamb. Beef fat contains 3.1–8.5 mg CLA/g, the 9-cis and 11-trans isomers contributing 57–85% of total CLAs (Decker, 1995). A significant increase in the CLA content of meat may result from cooking or processing; yet the obvious advantage of this from the standpoint of cancer prevention is likely to be at least partially offset by the fact that the concentrations of many mutagens and carcinogens increase as well.

2.3.15 Vitamins

Vitamins are defined as biochemical compounds required by the body in small amounts. There are two types of vitamins, water-soluble and fat-soluble ones. Fat-soluble vitamins, which include vitamins A, D, E, and K, are found mainly in fatty foods such as animal fats (including butter and lard), vegetable oils, dairy foods, liver, and oily fish. They are essential for proper functioning of the body. Excess amounts of vitamins derived from the foods are stored in the liver and fatty tissues for future use, but consumption of an excess can be very harmful. Water-soluble vitamins, which include vitamins B_6, B_{12}, biotin, folic acid, niacin, pantothenic acid, riboflavin, and thiamin as well as Vitamin C (Padh, 1994), are found in fruits, vegetables, and grains. As they are not stored in the body, these vitamins must be constantly supplied from dietary sources. In contrast to the fat-soluble vitamins, consumption of larger than essential quantities of water-soluble vitamins is generally not harmful, as the excess amounts are excreted in urine. As a rule, they are unstable when exposed to heat or air. When food is boiled in water, its water-soluble vitamins can be leached into the water and lost. Foods containing water-soluble vitamins

are best cooked by steaming or heating in a microwave oven to ensure retention of vitamins.

2.3.16 Minerals

Minerals are essential inorganic nutrients that the human body needs in small quantities and are often absorbed from food. Minerals are present in varying concentrations in a variety of foods, such as meat, cereals, fish, milk and dairy foods, vegetables, fruits (including dried fruits), and nuts. Minerals such as calcium and phosphorous are necessary for building strong bones and teeth, and sodium and potassium control the level of water inside and outside cells. Many minerals, including magnesium, iron, phosphorus, potassium, sodium, and sulfur, are enzyme cofactors and control the rate of enzyme reactions and energy production within the body (Anderson and Allen, 1994).

"Trace elements," such as boron, cobalt, copper, chromium, fluoride, iodine manganese, molybdenum, selenium, silicon, and zinc, are essential minerals that are needed in minute quantities compared with other minerals (Anderson and Allen, 1994). They are found in a variety of foods such as meat, fish, cereal, milk and dairy foods, vegetables, and nuts.

2.4 FUNCTIONAL FOODS AND NUTRACEUTICALS: HEALTH CLAIMS AND BENEFITS

2.4.1 Oats

There is evidence that oat β-glucan, like many other polysaccharides, may reduce total and LDL cholesterol, thereby reducing the risk of CHD (Wood and Beer, 1998). Oats contain β-glucan, which is one of the dietary sources of cholesterol-lowering soluble fiber and has been studied extensively. In support of a petition for a health claim, the Quaker Oats company (Chicago, IL) conducted clinical trials on the effects of oat consumption by humans between 1980 and 1995. This study revealed that statistically significant reduction in total cholesterol and LDL cholesterol was achieved in hypercholesterolemic subjects by daily consumption of 34–123 g of oat bran or oatmeal. It also showed that 3 g of β-glucan is required to obtain a 5% reduction of serum cholesterol, meaning that approximately 60 g of oatmeal or 40 g of oat bran had to be consumed. In order to qualify for a health claim, an oat product must contain 13 g of oat bran or 20 g of oatmeal, and provide at least 1.0 g of β-glucan per serving without any fortification (Hasler, 1998). On the basis of these studies, the FDA awarded the first food-specific health claim in January 1997 for oats and oat products.

2.4.2 Psyllium

The FDA extended the soluble fiber health claim to psyllium fiber in February 1998. Psyllium husk, which is marketed as Metamucil, is also used as an additive in several other products such as cereals, supplements, and soluble fiber in the diet. It confers several health benefits, notably alleviation of constipation and a moderate decrease in blood pressure.

2.4.3 Soybeans

Besides being a source of high-quality protein, soybean lowers cholesterol levels and reduces the risk of developing CVD. Soybeans and soy foods contain many phytochemicals that are considered to be effective in preventing and ameliorating many chronic diseases such as cancer, CVD, and osteoporosis, and it alleviates menopausal symptoms (Messina et al., 1997). The phytochemicals in soybeans include phytoestrogens (e.g., genistein, daidzein, and glycitein, and their glucosides), phytosterols, tocophenols, saponins, phenolic acids, lecithins, protease inhibitors, and phytic acid. The cholesterol-lowering effect of soybean has been well documented. A meta-analysis (1995) of 38 separate studies involving 743 subjects revealed that consumption of soy protein (25 g/day) resulted in significant reduction in total cholesterol (9.3%), LDL cholesterol (12.9%), and triglycerides (10.5%), with a small increase in HDL cholesterol (2.4%) (Anderson et al., 1995). However, isoflavones in soy protein did not seem to have any cholesterol-lowering effect (Nestle et al., 1997; Hodgson et al., 1998). The exact mechanism whereby soy protein exerts its hypocholesterolemic effect has not yet been fully elucidated, but may involve enhanced synthesis of the LDL receptor and inhibition of cholesterol biosynthesis (Cho et al., 2007). Recently, a meta-analysis of the relationship between soy isoflavones and LDL and HDL cholesterol concentrations in humans showed decreased LDL cholesterol and increased HDL cholesterol during isoflavone consumption (Weggemans and Trautwein, 2003). Yet, there was no dose–response relation between soy isoflavones and changes in LDL or HDL cholesterol levels, even though the study was carried out by feeding the subjects 36 g of soy protein per day together with 52 mg of soy isoflavones on the average. In November 1999, the FDA approved a health claim for soy protein on the grounds that daily consumption of at least 25 g of soy protein tends to reduce the risk of CVD.

Among the soybean phytochemicals that have anticarcinogenic effects, isoflavones (phytoestrogens) are the most noteworthy because soybeans are the only known dietary source of significant amounts of these compounds (Messina and Barnes, 1991; Hendrick and Murphy, 2000). There are 12 forms of isoflavonoid components. These include three aglycones (daidzein, genistein, and glycitein), three glucosides of aglycones (daidzin, genistin, and glycitin), three acetyl ester glucosides (acetyl-daidzin, acetyl-genistin, and acetyl-glycitin), and three malonyl ester glucosides (malonyl-daidzin, malonyl-genistin, and malonyl glycitin) (Jackson et al., 2002). Isoflavones are heterocyclic flavonoid components structurally similar to estrogenic steroids. Owing to their weak estrogenic activity, isoflavones may act as antiestrogens by competing with the more potent, naturally occurring endogenous estrogens (e.g., 17β-estradiol) for the estrogen receptor, or more likely, interfere with estrogen receptor action and signal transduction processes. This might be the reason why the populations that consume large quantities of soy products daily (e.g., inhabitants of Far Eastern countries) generally have a reduced risk of estrogen-dependent cancers (Messina et al., 1997).

Soybean and soy foods may also benefit bone health (Anderson and Garner, 1998; Messina et al., 2000). A clinical study of 66 postmenopausal women conducted at the University of Illinois (Erdman and Potter, 1997) demonstrated that consumption of 40 g of isolated soy protein (ISP) containing 90 mg total isoflavones per day increased both bone mineral content and density in the lumbar spine by ~2% over a period of 6 months. Furthermore, Far Eastern women are reported to have significantly lower incidence of

postmenopausal hot flushes and night sweating compared to Western women, probably because soy food is an important part of their diet (Albertazzi et al., 1998). Yet the suggestion that soy food consumption may substitute for hormone replacement therapy (HRT) may require further study. Recent research has shown that not only soybean seeds, but also soybean leaf powder and ethanol extracts of soybean leaf, exert cardioprotective effects by modulating serum lipid profiles (Ho et al., 2003).

2.4.4 Phytosterols

The FDA expanded the scope of a phytosterol heart health claim in February 2003. The agency allowed a broader range of food products and dietary supplements to bear the heart health claim in labeling when they are formulated with 0.65 g of phytosterol ester or 0.4 g of free phytosterol per serving.

2.4.5 Fiber

The FDA adopted a final rule to a regulation authorizing a health claim for the role of β-glucan from whole oat sources in reducing the risk of CHD in July 2003 (FDA, Federal Register of July 28, 2003 [68FR 44207-44209]).

2.4.6 D-Tagatose

Tagatose is a naturally occurring sweetener derived from fructose. By comparison to sugar which provides 4 kcal/g, tagatose is a low-calorie sweetener providing 1.5 kcal/g. It is not readily metabolized unlike sugars and therefore does not cause a sudden increase in blood sugar. It may be useful as a sweetener for type II diabetic patients, but may cause stomach upsets. The FDA authorized a dental health claim for D-tagatose in July 28, 2003 (FDA, Federal Register of July 3, 2003 [68 FR 44207-44209]).

2.5 QUALIFIED HEALTH CLAIMS

In July 2003, the FDA began allowing qualified health claims for food in an effort to make it easier for food manufacturers to make health benefit claims for their products. Even if the scientific evidences in support of the claims are not conclusive, the FDA has been helping the consumers to obtain accurate, up-to-date science-based information about the health effect of these products. Qualified health claims have been approved for a number of foods, and conditions for their use have been specified (www.cfsan.fda.gov). Such foods and their nutraceutical components, and diseases against which they appear to be effective, include the following.

2.5.1 Selenium and cancer

Selenium may help to prevent certain kinds of cancer. However, the FDA has determined that the evidence for this is limited and inconclusive. Eligible foods are dietary supplements containing selenium.

2.5.2 Antioxidant vitamins and cancer

Some scientific evidence suggests that consumption of antioxidant vitamins may reduce the risk of developing certain kinds of cancer. However, the evidence is increasing, and further insights may come through future studies. Eligible foods are dietary supplements containing vitamin E and/or vitamin C.

2.5.3 Nuts (e.g., walnuts) and heart disease

Scientific evidence suggests, but does not prove, that eating about 46.51 g (1.5 oz) per day of most nuts as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. Eligible foods are whole or chopped almonds, hazelnuts, peanuts, pecans, pine nuts, pistachio, Brazil nuts, and walnuts, or foods containing one or more of these nuts.

2.5.4 Omega-3 fatty acids and CHD

Consumption of omega-3 fatty acids may reduce the risk of CHD. The FDA evaluated the data and determined that, although there is scientific evidence supporting the claim, further studies will be helpful in substantiating the claim. Eligible foods are dietary supplements containing the omega-3 long-chain polyunsaturated fatty acids EPA and/or DHA.

2.5.5 Phosphatidylserine/Phosphatidylcholine and cognitive dysfunction and dementia

Consumption of phosphatidylserine may reduce the risk of dementia in the elderly. However, the FDA concludes that there is little scientific evidence in support of this claim. Eligible foods are dietary supplements containing phosphatidylserine. Consumption levels of phosphatidylcholine at 550 mg/day for men and 425 mg/day for women have been recommended. Phosphatidylcholine has been observed to be beneficial for maintaining cognitive function, protecting the liver and improving the balance of lipids in favor of "good cholesterol," providing a cardiovascular protective function.

2.5.6 Folic acid and neural tube birth defects

A 0.8-mg folic acid in a dietary supplement is more effective in reducing the risk of neural tube defects than lower amounts present naturally in foods. Folic acid has been recommended both by NIH (http://ods.od.nih.gov/factsheets/folate.asp) and the Government of Canada (www.hc-sc.gc.ca/fn-an/pubs/nutrition/folate-eng.php).

2.6 FUNCTIONAL FOODS AND NUTRACEUTICALS: SAFETY ISSUES

As functional foods and nutraceuticals are being marketed in ever-increasing quantities for human consumption, it is absolutely essential to ensure that they are safe and that they meet high standards of quality. The biologically active components have to be identified and quantified, and the manufacturers must strive for consistency of product quality and

levels of active components in the products. Above all, clinical studies must verify the safety and efficacy of the products, and must establish the proper effective dosage for the treatment of particular diseases and conditions. The optimal dosage has to be defined, as the dosage must be high enough to be effective, yet not so high that it is hazardous or causes adverse side effects or interacts in undesirable ways with other functional foods or nutraceuticals, or with prescription or nonprescription drugs.

It is highly advisable to consult a physician knowledgeable in the area, before planning to take any nutraceuticals or herbal supplements, especially if one has certain medical conditions, such as high blood pressure, thyroid dysfunction, psychiatric disorders, Parkinson's disease, blood clotting problems, diabetes, heart disease, epilepsy, glaucoma, and a history of stroke. Furthermore, the consumers should not assume that functional foods and nutraceuticals are safe merely because they are labeled "natural." It is important to recognize the fact that (1) functional foods and nutraceuticals may have harmful side effects in at least some individual cases, and that (2) these products may tend to cancel or amplify the function of a prescription drug or over-the-counter drug, causing adverse health effects (American Academy of Family Physicians, 2003; Mayo Clinic Commun., 2003). The standards for the quality of herbal health products are increasing, and government regulations pertaining to product safety and standardization of quality and concentrations of active components are becoming a common norm.

Some examples of the problems that may arise through consumption of functional foods or nutraceuticals/phytochemicals along with drugs are as follows (American Academy of Family Physicians, 2003; Mayo Clinic Commun., 2003).

2.6.1 Echinacea

Avoid taking echinacea with anabolic steroids or medicines for treatment of irregular heartbeat, rheumatoid arthritis, or fungal infections. Echinacea should not be combined with certain other drugs, as it may (1) cause liver damage, (2) stimulate the immune system and interfere with effects of immunosuppressants, (3) raise the levels of HIV protease inhibitors and calcium channel blockers, or (4) produce undesirable side effects when combined with antianxiety drugs.

2.6.2 Ephedra (also called "ma huang, herbal ecstasy, or mahuanggen")

Avoid taking Ephedra products with caffeine, decongestants, stimulants, heart drugs, antidepressants, or asthma inhalers. They can cause high blood pressure or uneven heartbeat, nervousness, or headache, and can lead to potentially fatal heart attacks or strokes.

2.6.3 Feverfew

Avoid taking feverfew with aspirin or other medicines that interfere with the clotting of blood. Feverfew may enhance this effect, causing spontaneous and excessive bleeding.

2.6.4 Garlic

Avoid taking garlic with aspirin or other medicines that interfere with the clotting of blood, or with immunosuppressants, or HIV protease inhibitor. Combining garlic and

anticoagulant medication may cause spontaneous and excessive bleeding. Besides, garlic may decrease the effectiveness of immunosuppressants and HIV protease inhibitors.

2.6.5 Ginger

Avoid taking ginger with aspirin or other medicines that interfere with the clotting of blood or with H2 blockers or acid-blocking medication. Ginger may increase the effect of anticoagulants and also increase the production of stomach acid, which would counteract the effect of anti-acid medications. On the other hand, ginger may lower the blood pressure or blood sugar levels, which may reduce the need for blood pressure-lowering medicines or insulin.

2.6.6 *Gingko biloba*

Avoid taking *G. biloba* with aspirin or other medicines that interfere with the clotting of blood or with antidepressant, antipsychotic medicines, or insulin. It may increase the anticoagulant effect of these drugs and can cause bleeding as a side effect. *G. biloba* can also increase the levels of antidepressant drugs in the blood. When combined with antipsychotic drugs, it may cause seizures. In addition, it may affect the insulin levels.

2.6.7 Ginseng

Avoid taking ginseng with aspirin or other medicines that interfere with the clotting of blood or with antidepressants, heart medication, insulin, or oral antidiabetic medicines. Ginseng combined with warfarin (coumadin) can increase the risk of excessive spontaneous bleeding. It can also interfere with digoxin's pharmacological action. Moreover, it can reduce blood sugar levels in people with type 2 diabetes (requiring especially careful glucose level monitoring).

2.6.8 Kava kava products

Avoid taking kava kava products with sedatives, sleeping pills, antipsychotic drugs, alcohol, or drugs for anxiety or Parkinson's disease. When combined with any of these drugs, kava kava can induce deep sedation and, in some cases, even coma. Liver damage resulting from the use of kava kava was reported from Europe in late 2001. The FDA undertook an investigation of the product's safety.

2.6.9 St. John's Wort

Avoid taking St. John's Wort with alcohol or with any prescription medicines such as antidepressants, digoxin, asthma medication, oral contraceptives, blood-thinning medication, Tamoxifen, drugs for controlling blood pressure, or medicines for heart disease. St. John's Wort affects the body's metabolism of these substances.

In conclusion, the benefits and risks of consuming the physiologically active functional foods and nutraceuticals have to be balanced carefully because (1) biological responses may differ widely among different individuals, (2) adverse effects are possible in some

cases, and (3) these substances may interfere with the effects of prescription or nonprescription drugs or other functional foods and nutraceuticals. Systematic regulation and quality control of functional foods and nutraceuticals marketed for human consumption are essential to public health and well-being.

2.7 REGULATION OF FUNCTIONAL FOODS AND NUTRACEUTICALS

There has been growing recognition of the key role of diet in disease prevention and treatment. Thus, the production and consumption of functional foods and nutraceuticals, the popular demand for them, and realization of their importance in promoting good health, have increased greatly in recent years. Accordingly, for manufactures and consumers alike, there must be well-defined, science-based standards and regulations to ensure that these products have consistently high quality, that their components are identified and accurately quantified, and that they are safe (Stephen, 1998). Product labels must list not only the components and their concentrations but also warnings about possible adverse effects and interactions or interference with other substances, such as drugs and food supplements. In addition, stringent clinical trials must be performed to investigate the beneficial effects, efficacy, and adverse effects of the products, to establish the proper dosage for the treatment or prevention of specific medical conditions, to determine the range of doses that are safe, and to study the consequences of combining them with drugs and other substances.

There have been several new developments with regard to the regulatory policies of functional foods and nutraceuticals in several countries. For instance, in Canada, Health Canada oversees the regulation of functional food and nutraceutical industry, with the Canadian Food Inspection Agency responsible for the implementation of protocols and standards. The "Food Directorate," an integral wing of Health Canada's "Health Products and Food Branch," regulates the functional foods, while the "Natural Health Products Directorate" regulates the nutraceuticals and other natural health products (www.hc-sc.gc.ca/fn-an/index-eng.php) while in the United States, the terms functional foods and nutraceuticals have no legal significance, and are regulated under the "Dietary Supplements Regulations" (Dietary Supplements Health Education Act of 1994, DSHEA) enforced by the FDA (www.fda.gov/Food/LabelingNutrition/FoodLabelingGuidanceRegulatoryInformation/default.htm and the www.fda.gov/Food/DietarySupplements/default.htm). The Federal Trade Commission regulates aspects related to the advertising, labeling, and marketing of dietary supplements (www.ftc.gov/bcp/policystmt/ad-food.shtm and www.ftc.gov/bcp/edu/pubs/business/adv/bus09.shtm). The "Food Safety and Inspection Services" of the United States Department of Agriculture are also involved in food quality regulations (www.fsis.usda.gov/About_FSIS/labeling_&_consumer_protection/index.asp).

The regulatory system of *Japan* has been supportive of the development and marketing of functional foods. The Ministries of Agriculture and Health and Welfare joined forces to develop a comprehensive policy to promote the manufacture of food components that confer potential health benefits. This policy is rooted in traditional Oriental lore which holds that many foods and food constituents are effective in the prevention and treatment of disease. Accordingly, in 1991, the government established a system called FOSHU for the purpose of licensing such substances (Stephen, 1998). Their objective, above all, was

to maximize the health of the growing numbers of elderly people in the population (Lapsley, 1996). It has evolved through close collaboration among food industries, government, academics, and research organizations. Thus, accredited food industry organizations work closely with government agencies and food companies, and play a direct role in the regulation of functional food products under the regulations of FOSHU. The licensing process is clearly defined and is managed jointly by government and industry. As a result, a functional food is evaluated, and food manufacturers may make health claims for it. On being licensed and receiving an official FOSHU seal of approval, these foods may then be sold on the domestic market, and consumers have confidence in them. Currently, there are over 150 FOSHU-approved products available in Japan.

In the 15 nations of the *European Union* there are a number of initiatives concerning functional foods, but they appear to concentrate mainly on relevant scientific issues (Smith et al., 1996; Stephen, 1998). Currently, regulation of functional foods, food supplements, and/or nutraceuticals is voluntary and is therefore left to the discretion of each member country. There are no universal standards, regulations, or policies pertaining to such products. Therefore, the systems of regulation vary widely from one member nation to another, and many countries consider these products simply as foods. Claims relating to a disease are generally not permitted. If such claims are made, however, the products come under the regulations for drugs and other medicinal substances.

In the *United States*, there is no separate category or set of regulations for functional foods and nutraceuticals. These products fall under the regulations for conventional foods. They must be safe to be marketed as foods, and the ingredients must be "generally recognized as safe" (GRAS) or approved as food additives. Nevertheless, there is a distinction between dietary supplements and foods. For a new ingredient to be approved for use as a dietary supplement, it is submitted to the FDA 75 days before the supplement is to be put on the market. FDA then evaluates it and either accepts or rejects it on the basis of safety data provided by the producer. Where foods are concerned, however, if an ingredient is not already GRAS, it takes a long time to be declared GRAS. There are no definite rules to guide this process and no clear standards for judging the data provided by companies. Having functional foods approved as GRAS is not a simple task.

Regarding botanicals used as food ingredients, the fundamental issues are the identity of the plant and identification and quantification of the phytochemicals in the plants. To settle the first issue, harvesting the correct genus of the plant is essential.

In *Australia*, the Parliamentary Secretary to the Minister for Health and Ageing Consumers recently released a policy statement called Food Standards Australia-New Zealand (FSANZ)-Food Labeling Issues after a survey of consumers in Australia and New Zealand. The key findings of the survey were that the most commonly examined specifications on the labels were the "by" or "best before" dates, lists of ingredients, and information about health claims, allergens, genetically modified organisms (GMOs), and novel or irradiated food declarations. The research also revealed that consumers want further information to help them to understand the basics of healthy eating and to make healthy choices of foods by following the guidelines provided by food labels. As a result, FSANZ has undertaken to work with the food industry, health professionals, and educators to ensure that this information is available. This survey was very timely, as it was conducted just before December 2002, when the New Food Standards Code came into full force (www.foodstandards.gov.au). Detailed compilations of regulatory issues for the functional foods and nutraceuticals have recently become available (Hasler, 2007; Bagchi, 2008).

2.8 PUBLIC EDUCATION AND DIETARY GUIDANCE

As a party to the Consumer Health Information for Better Nutrition Initiative of the U.S. government, the FDA is seeking ways to promote inclusion of dietary guidance statements on food labels. The agency is encouraging good nutrition among consumers in a variety of ways, including food manufacturers' dietary guidance statements, which are available from federal government agencies. The National Cancer Institute (NCI), in a collaborative effort with the FDA, recently issued an important dietary guidance message for consumers: "Diets rich in fruits and vegetables may reduce the risk of some types of cancer and other chronic diseases." Food manufacturers may also use this statement on food labels along with the new "5-to-9-Day" logo. The FDA has pointed out that this statement about fruits and vegetables is only a "dietary guidance statement" and not a "health claim," emphasizing, however, that dietary guidance statements must be truthful and not misleading. The FDA works cooperatively with federal agencies on an ongoing basis to encourage the development and use of dietary guidance messages (U.S. FDA, 2003). An example is FDA's small entity compliance guide (SECG) for a final rule on food labeling published in the Federal Register of July 11, 2003, entitled "Trans Fatty Acids in Nutrient Content Claims, and Health Claims." (Alternatively, this SECG is called "Food Labeling—Trans Fatty Acids in Nutrition Labeling, Nutrient Content Claims and Health Claims" (FDA, Federal Register, 68 (161), 50155–50156).

The average American eats over 4 g of salt a day. In order to reduce possible harmful effects of hidden salt in food, the National Institute of Health (NIH) advises healthy people to have no more than 2.4 g of salt (as sodium chloride) a day and 1.5 g for those with high blood pressure. Salt is concealed in common foods, especially restaurant foods and processed foods. Too much sodium increases blood pressure, and high blood pressure has adverse effects on the heart, brain, and kidneys. The American Public Health Association, backed by many other health-care and medical groups, urge the processed food industries and restaurants to lower the amounts of salt in their foods.

Another example of hidden food components that may be harmful is gluten, a protein found in wheat and related cereal grains. About 10–15% of the American population cannot tolerate gluten; indeed, gluten is actually toxic to some individuals. Today's consumers must check food labels to detect hidden gluten. They must be more cautious than ever nowadays, as gluten is found in more than 40,000 supermarket products, compared with 7000 products in the 1960s (www.glutenfree101.com).

A survey done jointly by the Food Marketing Institute (FMI) and *Prevention* magazine revealed that the choice of food purchases made by families with children depends mainly on convenience. Yet most consumers would like to have more information that will help them to eat more healthful foods and have a balanced diet (www.ift.org/ and www.fmi.org/). In the United States, nearly 65% of adults and 15% of young people aged 6–19 are overweight, and 31% of the adults are classified as obese according to the National Center for Health Statistics of the Centers for Disease Control and Prevention. An even more alarming fact is that the estimated number of overweight children and adolescents has nearly tripled in the past two decades according to the U.S. Surgeon General. The new survey showed that 74% of households with children and 63% with no children admit that their diets could be healthier. The survey results revealed the following primary reasons that the subjects did not have healthier diets: (1) They were "too busy" to eat healthy foods, they had no time to cook at home, and a third of the women worked outside the home; (2) "Healthy

fast foods are hard to find." One-third of shoppers would like to see a greater variety of healthy foods on the menus of fast-food restaurants; (3) "Healthy foods cost too much"; (4) Friends and family members "do not care" about healthy eating habits, educating friends and family members about nutrition and the necessity of eating balanced meals to maintain good health is important; and (5) There is "confusion about health claims," consumers are confused about what qualifies as a healthy food product and what they should eat to have healthy and balanced diet (www.fmi.org/).

2.9 CONCLUDING REMARKS

In recent years, there has been enormous progress in the study of functional foods and nutraceuticals and their importance to the health of humans and animals, but there is a need for further research in many areas of this ancient, yet modern, field. To begin with, there is ample scope for more basic research that will lead to a better understanding of the mechanisms whereby functional foods and nutraceuticals exert their beneficial (and, in certain cases, harmful) effects. This, in turn, should lead to advances in the applied aspects of the field. Thus, the biologically active components must be identified and quantified using rigorous, standardized, internationally accepted methods. Concomitantly, clinical and epidemiological investigations are required to assess the effects of functional foods and nutraceuticals on human health, with attention not only to their efficacy but also to issues such as proper dosage, delivery methods, bioavailability, and safety (e.g.,, possible adverse effects arising from allergies or interference with prescription or over-the-counter pharmaceuticals or with other functional foods and nutraceuticals). Another important research area is exploration for the purpose of discovering as yet unknown medicinally beneficial substances in plants and other organisms (e.g., marine algae and animals). It is essential, however, that the discovery and subsequent exploitation of new sources of functional foods and nutraceuticals in nature be accomplished without damaging the environment or depleting populations of wild species.

Furthermore, the field of functional foods and nutraceuticals has political, legal, and administrative dimensions. Thus, laws and government policies, regulations, and guidelines must be established to authorize the marketing, labeling, and advertising of specific products on the basis of scientifically sound criteria for evaluating their efficacy, safety, and quality. The field is economically important as well. The production, processing, and marketing of functional foods are a thriving, multifaceted business. The sale of such products has expanded greatly in the recent past to meet rising public demand for such products as a consequence of increasing awareness of their beneficial effects on human health. A less direct but equally significant economic issue is the reduction in the cost of health care that may be expected if public health is benefited by improvements in eating habits.

Finally, public education about functional foods and nutraceuticals is another vitally important aspect of this field. A healthful diet helps to prevent and ameliorate the chronic diseases which are so prevalent in modern society. Yet poor eating habits and their inevitable harmful consequences constitute a serious, widespread problem. The more thoughtful members of the general public have become increasingly concerned about dietary issues, increasingly aware of the crucial importance of a balanced, healthful diet, and increasingly knowledgeable about the good health and improved quality of life that may result from regular consumption of functional foods and nutraceuticals.

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3 Nutritional Genomics: Fundamental Role of Diet in Chronic Disease Prevention and Control

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3.1 INTRODUCTION

Nutritional scientists have long acknowledged and intensively studied the relationship between the presence and abundance of specific nutrients and non-nutritive compounds in the diet of various populations, and the incidence of chronic disease. However, the molecular basis for these correlations and variability in response among individuals has long eluded our complete understanding. The advent and integration of technologies permitting the characterization of DNA sequence and chromatin modifications, as well as rapid and accurate quantification of messenger ribonucleic acid (mRNA), protein, and metabolite levels, has steadily permitted the refinement of our understanding for the biological activities of nutrients and non-nutritive compounds, including the basis for interindividual differences in response to their consumption. Although the disciplines, applicable technologies, and specific terms for the various branches can vary considerably, the concept of "nutritional genomics" has emerged from this quest for identifying the mechanistic links between diet, genetic profile, gene expression, metabolic phenotype, and disease.

The term nutritional genomics refers to the study of the bidirectional interactions between specific nutrients/non-nutritive compounds and cellular function (Rimbach and De Pascual-Teresa, 2005). As will be illustrated here, these interactions can be observed at multiple levels, including the genome (i.e., the entire set of genes present in a given organism), the epigenome (i.e., the collection and pattern of potentially heritable chromatin modifications that influence gene expression, independent of DNA structure), the transcriptome (i.e., the full complement of RNA products transcribed in a given tissue/cell type), the proteome (i.e., the global set of translated and posttranslationally modified proteins in a given tissue/cell type), and the metabolome (i.e., all low-molecular-weight metabolites found within a given biological sample). Thus, the study of these interactions has progressively contributed to the development of many new fields and frontiers within nutritional sciences, including

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1. Nutrigenetics—the study of how individual genetic variability determines unique responses to dietary factors and the risk of developing diet-related diseases (Zhang et al., 2008);
2. Nutriepigenetics—the study of the influence which both prenatal and perinatal diet can exert over epigenetic modifications to chromatin structure, and the associated effects on gene expression (Gallou-Kabani et al., 2007a);
3. Nutritranscriptomics (aka nutrigenomics)—the study of the changes to transcriptional activity in response to specific dietary factors through mRNA quantification and analysis (Mutch et al., 2005b; Zhang et al., 2008);
4. Nutriproteomics—the study of the effects of dietary factors on protein synthesis, posttranslational modifications, and function (Barnes and Kim, 2004; Zhang et al., 2008); and
5. Nutrimetabolomics—the study of the profile and function of the metabolome associated with nutritional status and/or intervention (Gibney et al., 2005; Zhang et al., 2008).

Together, these approaches contribute to an understanding of how an individual genetic profile determines the effects of nutrients, and conversely, the multilevel intracellular effects of nutrients on DNA structure, mRNA abundance, protein function, and metabolite profile. Bioinformatic tools have increasingly permitted the comprehensive analysis and integration of data sets from each level of nutritional genomics. These tools promise a system-wide understanding of the response to nutritional intervention and potentially will yield novel, highly sensitive biomarkers, indicative of the preclinical state for diet-induced chronic diseases (Muller and Kersten, 2003). This chapter will largely focus on recent findings in the realm of nutrigenetics, nutriepigenomics, and nutritranscriptomics, and their relation to diet-related chronic disease, with an emphasis on those examples pertinent to cardiovascular disease (CVD) and cancer. As a preamble to these discussions, fundamental concepts and methodological challenges within each of the respective areas of research will be briefly addressed. Excellent reviews on the scientific promise and unique barriers facing nutriproteomics (Barnes and Kim, 2004) and nutrimetabolomics (Gibney et al., 2005) are available elsewhere.

3.2 NUTRIGENETICS

3.2.1 Gene polymorphisms

The powerful influence which genetic variability can exert in determining an individual's susceptibility to disease has been firmly established, with many forms of classical monogenic disorders now documented. Common examples include phenylketonuria, galactosemia, lactose intolerance, celiac disease, and familial hypercholesterolemia (Ordovas and Corella, 2004). Such disorders are traditionally thought to be related to mutations in single genes, resulting in impaired or nonfunctional protein products, critical to metabolic pathways which display dysregulation the aforementioned disease states.

This is in contrast to the multifactorial chronic diseases, such as CVD and cancer, where the phenotype is thought to be elicited by multiple genetic and environmental factors (Manolio et al., 2008). Hereditary mutations predisposing to disease can vary considerably in functional significance and prevalence, a notion well illustrated in the case of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Point mutations,

both missense and nonsense, frameshift mutations, splice-site variants, and even larger scale deletions of the *CFTR* gene have all been shown to be associated with the incidence of cystic fibrosis (Tsui, 1992). However, the most prevalent of these mutations, a 3 bp deletion resulting in the removal of a phenylalanine residue at codon 508 (ΔF508), has been found to occur at a relative frequency greater than 67% (Tsui, 1992). Mutations such as this, in which the least common allele at a given locus occurs at a frequency of 1% or greater, are typically referred to as polymorphisms.

3.2.2 Single nucleotide polymorphisms (SNPs)

As a genetic component in the pathophysiology of chronic disease, polymorphisms can contribute to susceptibility and progression of both CVD (Anderson et al., 2007) and various forms of cancer (Bernig and Chanock, 2006). Polymorphisms also come in various forms, including microsatellite repeat sequences, copy number variations, and SNPs. Of these, SNPs are the most common, accounting for approximately 90% of human DNA polymorphisms, and are the most simple. A SNP describes an individual substitution within the DNA sequence, resulting from either nucleotide transition or transversion, which varies between individuals of the same species (Ordovas and Corella, 2008). More than 10 million SNPs may exist at a frequency greater than 1% of the population, and some common SNPs are found in 5–50% of the population (McVean et al., 2005). The link between SNPs and chronic disease is typically evaluated using either candidate gene (hypothesis driven) or genome-wide (hypothesis-generating) association studies, with each approach imposing unique methodological challenges (Coassin et al., 2009; Jorgensen et al., 2009). Beyond the daunting task of SNP discovery and validation in varying cohorts and ethnicities, where enormous leaps of progress have been made in the last decade (Sherry et al., 2001; International HapMap Consortium et al., 2007), lies the challenge of determining the functional significance of these genetic variants. As some SNPs may ultimately have either null or negligible effects on the proper expression of a given protein, in a functional, temporal, and spatial sense, but still be in linkage disequilibrium (LD) with other functionally important SNPs, it is possible to identify misleading associations between a given variant and disease in one ancestral population, and not be able to reproduce the finding in another (Neale and Sham, 2004). Therefore, characterization of functional versus nonfunctional SNPs, as a prerequisite for progressing with association studies, has been increasingly pushed for by molecular epidemiologists (Humphries et al., 2004). Accordingly, the following section will introduce the various forms of functional SNPs, prior to addressing the interaction of genetic variability and environmental factors.

3.2.3 Nonsynonymous single nucleotide polymorphisms (nsSNPs)

When nsSNPs fall within exon sequences of genes encoding cellular enzymes, transporters, and receptors, expression may result in a gain-of-function, defective, or even nonfunctional protein products (Prokunina and Alarcon-Riquelme, 2004). A classical example of this effect is the methylene tetrahydrofolate reductase 677C>T SNP, which results in the substitution of an alanine residue for valine at codon 222 and the expression of a thermolabile enzyme (Engbersen et al., 1995), thereby impairing folate metabolism and potentially contributing to various CVD risk factors and the risk for colon cancer (Blom, 1998; Koushik et al., 2006). De Vos and colleagues recently explored the effects of 13

synonymous and nsSNPs (7 from coding sequences) in multiple genes involved in folate uptake and metabolism, on blood folate and homocysteine levels, as well as DNA uracil incorporation (DeVos et al., 2008).

3.2.4 Regulatory single nucleotide polymorphisms (rSNPs)

However, as described above, SNPs can reside in noncoding and regulatory regions of genes as well, in which case they are termed rSNPs (Prokunina and Alarcon-Riquelme, 2004). Those rSNPs residing in cis regions have the capacity to eliminate, modify, or create transcriptional-regulatory protein binding sites, resulting in altered protein–DNA interactions, and possibly altering the timing and level of gene expression (Prokunina and Alarcon-Riquelme, 2004). Furthermore, rSNPs located in cis positions have been shown to influence the epigenetic modifications (i.e., DNA methylation) within the promoter of the tumor suppressor gene O^6—methyl guanine methyl transferase (*MGMT*) (Candiloro and Dobrovic, 2009). As aberrant methylation of the *MGMT* promoter has been cited as a potential prognostic biomarker of glioblastoma and hepatocellular carcinoma (Lou et al., 2008; Gerstner et al., 2009), the clinical relevance of this variant should be further investigated. Those rSNPs located in 3′ untranslated regions of protein-coding genes can regulate mRNA stability, potentially through the alteration of microRNA (miRNA) binding sites, thereby attenuating or enhancing degradation via the RNA-induced silencing complex (Chen et al., 2008). Very recently, an SNP in the putative miRNA binding site of the estrogen receptor 1 gene displayed an association with breast cancer incidence in premenopausal women (Tchatchou et al., 2009).

3.2.5 Splice site single nucleotide polymorphisms (ssSNPs)

The extensive utilization of alternative splicing in the human genome also creates the possibility for ssSNPs to modify exon–intron boundary recognition, potentially leading to exon exclusion and/or the generation of novel splice variants (Prokunina and Alarcon-Riquelme, 2004). Indeed, Yang and colleagues have recently reported on the development and compilation of an ssSNP database for the human and mouse genomes, stressing the significant implications for these rSNPs in disease susceptibility (Yang et al., 2009).

3.2.6 *Trans*-Acting rSNPs

Finally, *trans*-acting rSNPs—those present in genes encoding factors involved in transcriptional regulation of target genes, can alter functionality of such proteins and thereby indirectly affect target gene expression patterns (Prokunina and Alarcon-Riquelme, 2004). For example, the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) gene, which encodes an important transcriptional activator that enhances activity of many nuclear receptors, is involved in the regulation of expression for many genes involved in cellular energy homeostasis. The Gly482Ser missense mutation in the PGC-1α gene was previously shown to be associated with reduced insulin sensitivity, an effect potentially mediated through downstream effects of this rSNP on PGC-1α target genes involved in hepatic gluconeogenesis, thermogenesis, and fatty acid (FA) oxidation (Fanelli et al., 2005), although *in vitro* analysis of the functional significance of this variant has provided contrasting results (Choi et al., 2006; Nitz et al., 2007). Beyond *in vitro* or *in vivo* determination of the effect of a given variant on protein expression and function, multiple *in silico*

approaches have been adopted for the prediction of deleterious mutations. They are largely based on basic principles of protein chemistry, three-dimensional structure, and species homology (Rebbeck et al., 2004), but not utilized without significant risk for erroneous prediction (Tchernitchko et al., 2004).

3.3 COMPLEXITIES OF CHRONIC DISEASE RESEARCH IN NUTRIGENETICS

In addition to considerations regarding SNP functionality, differing allele frequencies, and LD structure across ethnicities, population stratification and allelic heterogeneity—that is, variation in disease-associated allele within the same locus—can contribute to the generation of spurious associations or inconsistencies in the replication of a true association (Neale and Sham, 2004). Addressing the interplay of these additional complicating factors in genetic epidemiology for complex disease remains a contentious issue (Schork et al., 2009). On the one hand, the common disease-common variant (CDCV) hypothesis holds that genetic susceptibility to complex disease, such as CVD or cancer, is predominantly derived from multiple "old" variants with substantial population-level frequency, but low relative penetrance. This position implies that variation in the occurrence of complex disease among populations is largely reflective of disparities in environmental exposures. By contrast, the common disease-rare variant (CDRV) hypothesis contends genetic predisposition to complex disease is principally related to infrequently observed DNA sequence variations with greater relative penetrance (Schork et al., 2009). Under this premise, one assumes that certain predisposing variants will be largely restricted to specific populations who have experienced similar selective forces and genetic drift (Ordovas and Corella, 2006). The belief that one of these hypotheses has the capacity to yield greater insight into the genetic contribution ultimately guides the choice of methodology in a given association study. For instance, a CDCV approach might involve using haplotype data derived from the HapMap consortium, a compilation of over 3.1 million SNP locations derived from four populations, providing 25–35% coverage of the common SNP variation within these diverse cohorts (International HapMap Consortium et al., 2007), to conduct a genome-wide association (GWA) study linking a given variant with the incidence of complex disease in cases versus controls. Through the analysis of haplotype block diversity, the HapMap consortium has also identified tag SNPs—that is, representative SNPs that account genetic variability within specific genomic regions—due to high LD with other common variants in the same block (Manolio et al., 2008). This reduces the number of common SNPs which must be surveyed in GWA study, as carefully selected tag SNPs can be used to predict the remaining common variants in the same haplotype block. A spectrum of high-throughput genotyping technologies have rapidly evolved to compliment these approaches, with some chip applications now capable of accommodating greater than 1 million common SNPs (Ragoussis, 2009). Undoubtedly, these GWA approach have already yielded a bounty of statistically compelling insights into the genetic architecture of complex disease (Manolio et al., 2008). However, most common variants associated with complex disease identified thus far have displayed low penetrance and confer only minor increments of risk, with typical risk ratios between 1.1 and 1.5 (Manolio et al., 2009). This often leaves as much as 90–95% of the heritability (defined as the proportion of phenotypic variance accounted for by genetic variability) for complex diseases and traits unexplained by the additive effects of common variants, at least suggesting that they are not the predominant genetic

factors predisposing to complex disease (Schork et al., 2009). Furthermore, the common SNPs associated with complex disease using GWA methods may simply tag rarer causal variants, thereby convoluting the task of follow-up functional analysis (McCarthy and Hirschhorn, 2008). Although there have been strategies proposed to maximize the utility of the GWA method (Manolio et al., 2009), the so-called "missing heritability" provides a strong basis for the consideration of low-frequency and rare variants (CDRV hypothesis) in the etiology of complex disease. Low-frequency variants are typically defined as having a minor allele frequency (MAF) <5%, whereas rare variants have a MAF <0.5%. Unfortunately, their frequency prohibits inclusion in current GWA genotyping platforms, and their intermediate effect sizes are insufficient to be detected employing classical linkage approaches (McCarthy and Hirschhorn, 2008). However, there is a growing consensus that such variants may individually and collectively explain a greater proportion of the heritability of complex disease and disease-related quantitative traits than the common variants identified in GWA studies (McCarthy and Hirschhorn, 2008).

3.4 CHRONIC DISEASE AND RARE SNPS

Indeed, a growing body of research, in which candidate genes are resequenced among subjects so as to compare the frequency of rare variations among cases versus controls, has provided evidence that numerous complex diseases and disease-related phenotpyes are associated with rare SNPs in the coding regions of the genes studied (Schork et al., 2009). For instance, rare nsSNPs in the adenomatous polyposis coli (*APC*) gene were found to be significantly overrepresented among a cohort of North American subjects with colorectal adenomas, but without the conventional pathogenic *APC* or human MutY homologue mutations (Azzopardi et al., 2008). Furthermore, the functional significance of these rare SNPs were probed, as the authors evaluated their effect on *APC*-mediated suppression of β-catenin signaling, and later implicated the rare Glu1317Gln SNP for its novel role in contributing to colorectal tumorogenesis (Dallosso et al., 2009). Similarly, an enrichment of rare nonsynonomous sequence variants in the adenosine triphosphate binding cassette transporter A1 (*ABCA1*), apolipoprotein AI (*APOA1*), and lecithin cholesterol acytranserase (*LCAT*) genes was observed in those with low levels of high-density lipoprotein (HDL)-cholesterol (<5th percentile), a known risk factor for CVD, as compared to those with high levels of HDL (>95th percentile) (Cohen et al., 2004). A more complete understanding of the catalog of rare mutations at the level of the genome, as opposed to only candidate genes, will be necessary to fully evaluate their role in the genetic architecture of complex disease, an effort for which emerging sequencing technologies, namely massively parallel approaches (Tucker et al., 2009), and the "1000 genomes project," which will characterize genetic variation among 1000 individuals as a reference for further sequencing studies (Li and Leal, 2009), will play important roles.

3.4.1 Copy number variants

Ultimately, it is important to recognize that the CDCV and CDRV hypotheses are not mutually exclusive and that they can coexist as complimentary perspectives in genetic medicine. It is also important to note that other forms of potentially important differences in DNA structure exist among individuals, such as copy number variations (CNVs). CNVs are inherited or *de novo* deletions or duplications of a given DNA segment, thought to

potentially influence gene expression through a variety of mechanisms, depending on their origin and chromosomal location (Henrichsen et al., 2009). There has been a steady increase in the interest of their role in human phenotypic diversity and complex disease susceptibility (Wain et al., 2009), despite some initial results indicating null associations between >550 common and >8000 rare CNVs and myocardial infarction risk (Myocardial Infarction Genetics Consortium et al., 2009). Furthermore, complex gene–gene (epistatic) interactions can influence one's predisposition to disease, a phenomenon documented among SNPs within the AP0A1/C3/A4/A5 gene cluster, where 10 pairs of SNPs displayed nonadditive genotype effects on total cholesterol (Hamon et al., 2006). Epigenetic modifications, to be discussed in the subsequent section, also have the capacity to influence these parameters. Finally, the contribution of environmental factors cannot be overlooked.

3.5 CVD AND NUTRIGENETICS

In 1996, a total of 177 risk factors for CVD were identified and were subsequently grouped into 10 expansive categories. The vast majority of these risk factors could be considered related to one's environment in the broadest sense, and included 33 nutrition-related risk factors and another 34 related to drug, chemical, hormone, and nutritional supplement intake (which incorporated drug–drug and drug–food interactions) (Omura et al., 1996). In fact, this compilation of risk factors only included five deemed heritable, further stressing the predominant role which environmental factors, especially those related to diet, have traditionally assumed in the clinical conceptual framework of CVD pathophysiology. And yet, twin study data from the Swedish Adoption/Twin Study of Aging indicated the heritability of serum lipid levels (total and HDL-cholesterol, triglycerides, APOA1 and B) ranged from 0.28 to 0.78, whereas the environment of rearing accounted from 0.15 to 0.36 of the variance in total cholesterol (Heller et al., 1993).

3.6 NUTRIGENETICS AND CANCER

Similarly, dietary factors have been long cited as important contributors to the onset and progression of multiple forms of cancer. In the case of cancer, data from the Swedish, Danish, and Finnish twin registries suggested that environmental factors play the predominant role in the causation of 11 forms of sporadic cancer; however, heritable factors still accounted for large components of the risk for prostate, colorectal, and breast cancer (Lichtenstein et al., 2000).

3.7 SUMMARY OF NUTRIGENETIC RESEARCH POTENTIAL

These "unmeasured genotype" approaches, in which the frequency of specific variants remain undetermined, provide preliminary indications of the relative contributions of both hereditary and environmental factors in determining the risk of CVD and cancer. However, they provide no insight into the identity of the actual predisposing DNA sequence variants, or the role of specific environmental factors. As highlighted above, the enormous advances

in molecular genetics have allowed for direct measurement of variants in candidate genes, and the role of nutritional (environmental) risk factors has been established for both CVD and cancer. It is important to recognize that DNA sequence variants, and namely SNPs, do not directly cause common disease, as evidenced by the aforementioned low to intermediate effect sizes on CVD or cancer risk. It is the effects that they have on the functionality of their protein products that has the capacity to predispose certain individuals. Because many protein products are involved in the response to and metabolism of nutritional factors (both nutrients and non-nutritive phytochemicals), the possibility for gene–diet, or genotype–diet interactions emerges. The definition of interaction in this context can take both biological (alluded above) and statistical forms. In a statistical sense, interaction could be defined as a difference in the magnitude or direction of the effect exerted by a dietary factor (i.e., macronutrient, micronutrient, or phytochemical intake), depending on a given genetic factor (i.e., variants such as SNPs).

3.8 NUTRIEPIGENETICS

3.8.1 Role of the epigenome

Dietary nutrients significantly mediate gene expression via influence on the epigenome. The study of epigenetics encompasses both meiotically and mitotically inherited changes and has a key role in X-chromosome inactivation, genomic imprinting, and autosomal gene expression (Wilson, 2008). Epigenetic changes, or epimutations, are principally reversible and transient changes to the genome that may alter transcription but do not affect or change the primary DNA sequence (Choudhuri et al., 2010). Epimutations may be passed on to subsequent generations with potentially harmful effects (Wilson, 2008), predominantly for exposures during critical periods of periconception, fetal, and infantile development which may lead to increased disease susceptibilities later in life (Attig et al., 2010).

3.8.2 Cause of epimutations

Epimutations include DNA methylation, modification of histone tails of nucleosome cores, and alteration by noncoding RNA. The latter is primarily involved in changes via X-chromosome inactivation and genomic imprinting (Choudhuri et al., 2010) and will not be discussed here. Of relevance is DNA methylation, the covalent addition of methyl groups to specific sites of DNA, which has an inverse relationship with gene expression (Wilson, 2008). DNA methyltransferases catalyze these reactions, and methyl groups preferentially bind to cytosines 5′ of guanines, named CpG sites. For example, about half of the human genome has CpG islands, dense groups of cytosine and guanine dinucleotides, in the promotor regions of genes (Zeisel, 2007). The binding of methyl groups likely interferes with access of transcription factors to the promoter, thereby silencing gene expression (Wilson, 2008), and the timing of gene methylation is tissue and gene specific (Burdge et al., 2009). Histone proteins, H2A, H2B, H3, and H4 are present in duplicate with each 147 bp of DNA complexed to form a nucleosome (Wilson, 2008). The histone proteins are responsible for packing chromosomal DNA, but covalent posttranslational modifications lead to changes in nucleosome structure and accessibility of DNA for transcription. A change in charge from positive to negative via acetylation, methylation, phosphorylation, ubiquitination,

adenosine diphosphate (ADP)-ribosylation and sumoylation, and histone proteins will not bind as tightly to negatively charged DNA and thus, chromatin is less densely packed favoring gene expression (Wilson, 2008). Histone acetylation is the most common, and inhibitors of histone deacetylases may have a future role in disease therapy. Interestingly, DNA methylation and modification of histone proteins is inherently linked; methyl-CpG-binding proteins can recruit histone deacetylases, which further represse gene expression. Together, unique DNA methylation and histone modification patterns are termed epigenetic signatures and are essential in normal cellular differentiation.

3.9 EPIMUTATIONS IN CHRONIC DISEASE

Although understanding the normal epigenetic state will be a crucial first step in this field (Choudhuri et al., 2010), epigenetic signatures are also central to the control of disease processes. Research has found that epimutations may be involved in, but limited to, neoplastic, inflammatory, and autoimmune diseases, atherosclerosis, and hypertension (Torrens et al., 2006; Gallou-Kabani et al., 2007b; Wilson, 2008). Aging, similar to cancer, is associated with a clear impact on the epigenome, shown by a global decrease in CpG methylation, with specific regions of hypermethylation typically seen in promoter regions. Therefore, epigenome dysregulation may help to explain the increasing frequency of some late-onset chronic diseases (Wilson, 2008). To date, most of the research conducted involving nutrition status is centralized on maternal over- and undernutrition and its effects on offspring and their disease susceptibility later in life (Attig et al., 2010), which will not be reviewed here.

3.9.1 Epimutations and macronutrients/micronutrients

Epimutations may result from changes in dietary and metabolic precursors and cofactors for methylation and acetylation of DNA and histone proteins (Cooney, 2009). Nutrients involved in donating methyl groups or acting as cofactors in one-carbon metabolism for the methyl donor *S*-adenosylmethionine, including dietary folates, betaine, choline, zinc, and vitamin B12, are important regulators over DNA and histone methylation patterns (Cooney, 2009). Histone acetylation donor, acetyl-coenzyme A, is an intermediate in fat and carbohydrate catabolism; therefore, macronutrient balance may impact the production and transport of acetyl groups which also depends on regulatory molecules, cofactors, and vitamins including pantothenate (Cooney, 2009). In particular, nutrition in early life is crucial for the development of epigenetic signatures (Burdge et al., 2009). Well-known is the detrimental impact of folic acid deficiency during early pregnancy, associated with genomic hypomethylation, and the presence of open neural tube defects in the fetus (Wilson, 2008). In a similar way, folic acid supplementation in pregnant rats on a protein-restricted diet prevented the induction of hypertension and vascular function in the offspring (Torrens et al., 2006). Selenium supplementation has also been shown to modify the deleterious effects of folate deficiency and may also alter methylation patterns (Davis and Uthus, 2004). Dietary nutrients therefore are not acting exclusively and thus, it is evident that combinations and interactions of dietary nutrients must be considered in epigenetic research.

3.9.2 Epimutations and phytochemicals

Phytochemicals may alter the epigenome and be especially important in modifying disease risk. Soy or genistein exposure may modify breast cancer risk via epigenetic mechanisms. Genistein has been shown to affect DNA methylation by inhibiting DNA methyltransferases in animal models; however, it may also induce methylation with maternal exposure (Warri et al., 2008). Methylation patterns of key genes related to mammary tumorigenesis, BRCA1 and phosphatase and tensin homolog deleted on chromosome 10, may depend on the timing of exposure to soy/genistein (Warri et al., 2008), which is reflected in animal and human intervention studies as recently reviewed (Nagata, 2010). Tea polyphenols, including epigallocatechin-3-gallate (EGCG), a major polyphenol from green tea, have also been shown to inhibit enzyme activity of DNA methyltransferase and may reverse epigenetically silenced genes during carcinogenesis (Fang et al., 2005). To a lesser extent, bioflavonoids including quercetin, fisetin, and myricetin can also inhibit DNA methyltransferase-1-mediated DNA methylation (Lee et al., 2005), and lycopene can modify gene-specific methylation in breast cancer cells (King-Batoon et al., 2008). Histone acetylation is also influenced by phytochemical intake; for example, resveratrol and sulforaphane are both known inhibitors of histone deacetylases (Attig et al., 2010). Reseveratrol and its metabolites target NAD^+-dependent histone deacetylase SIRT1, which may be in part responsible for the chemoprotective properties of this polyphenol (Calamini et al., 2010). Sulforaphane is an isothiocyanate, which can effectively inhibit histone deacetylase and may have potential as a chemoprevention agent for prostate cancer (Ho et al., 2009). Overall, there are many potential therapies for modification of epigenetic signatures through nutritional interventions, especially for cancer prevention and treatment.

3.10 SUMMARY OF EPIGENETIC RESEARCH POTENTIAL

Epigenetic research is at the forefront of understanding human development and disease but still has many hurdles to overcome. It is important to recognize that early life exposures may help define susceptibility to disease, but disease risk may be modified by environmental exposures throughout childhood and adulthood. Primary therapies will likely target the use of natural foods and their purified bioactive compounds, such as soy/genistein and green tea/EGCG for nutritional interventions against cancer. Simultaneously studying normal epigenetic signatures under healthy circumstances will add immense value to the study of diseases in hopes to elucidate preventative and therapeutic treatments.

3.11 NUTRIGENOMICS

To explore gene expression on an integrated biological level is to study the emerging field of nutrigenomics. Nutrigenomics can be simplified into the effects that single or combinations of nutrients have on the regulation of gene expression (Muller and Kersten, 2003). The term remains unregulated in that it can also apply to the study of nutrigenetics, as discussed previously. To ameliorate this, a more specific term, nutritranscriptomics, was introduced by Zhang et al. in 2008 (Zhang et al., 2008) to clearly identify it as the study of the effect of dietary changes on gene transcription; however, the new term has not been widely adopted, and therefore, nutrigenomics will be used throughout.

3.11.1 Genomic impact of diet

Dietary factors have been compared to signals that can be identified and have influence over cellular sensors, including transcription factors, which may affect the genome at the level of mRNA or protein expression (Garcia-Canas et al., 2010). Modern technologies in molecular biology have allowed for simultaneous study of dietary factors on multiple genes and gene products and have allowed for exploration of novel nutritional biomarkers of disease (Mutch et al., 2005a). Goals of the field of nutrigenomics include to analyze these effects at the genome level in response to varying nutrients and bioactive compounds, to understand the molecular links between nutrition and physiology, and to potentially develop functional foods to prevent or manage chronic diseases (Hocquette et al. 2009; Simopoulos, 2010). In particular, nutrigenomics provides a balance between the contributions of genetics and diet in determining cancer and CVD risk (Iacoviello et al., 2008). Bioactive food components including glucose, cholesterol, FAs, vitamins A, E, D, phytoestrogens, phytosterols, and polyphenols may provide protection for these human chronic diseases.

3.11.2 Carbohydrates and gene interactions

3.11.2.1 Glucose and carbohydrate-responsive element-binding protein (ChREBP)

Understanding glucose metabolism is at the forefront of type 2 diabetes (T2D) research due to its influence over severity of disease, as well as risk for comorbidities including CVD. Of interest is the role that glucose has in lipogenesis, the process whereby excess dietary carbohydrates are converted into triglycerides for storage in adipose tissue. Although many transcription factors may be involved, including sterol regulatory element binding-protein (SREBP), liver X receptor (LXR), and farnesoid X receptor (FXR), the ChREBP is considered the most important. ChREBPs are known to control approximately 50% of hepatic lipogenesis by regulating glycolytic, gluconeogenic, and lipogenic gene expression (Iizuka and Horikawa, 2008). ChREBP mRNA is most abundant in liver, brown and white adipose, small intestine, kidney, and muscle (Iizuka and Horikawa, 2008). A member of the basic-helix-loop-helix (bHLH) leucine zipper family, ChREBP forms heterodimers with the bHLHZip protein Max-like protein X (Mlx) to bind to carbohydrate response elements (ChoRE) (Iizuka and Horikawa, 2008). The ChoRE consists of two E box sequences (CACGTG) separated by five base pairs and is the recognition site for ChREBP and Mlx (Davies et al., 2008). ChREBP is a transcription factor that preferentially regulates triglyceride storage, controlling the switch between lipogenesis through triglyceride and glycogen synthesis (Iizuka and Horikawa, 2008). With a high carbohydrate diet, excess carbohydrate is converted into triglycerides in the liver by key glycolytic enzymes such as glucokinase and liver-type pyruvate kinase (L-PK), acetyl CoA carboxylase (ACC), and fatty acid synthase (FAS) (Iizuka and Horikawa, 2008). Glucose regulation of ChREBP involves at least two distinct processes, an increased rate of nuclear entry and transition from a repressive to an active state (Davies et al., 2008), proposed to occur through glucose conversion to xylulose-5-phosphate-activating protein phosphatase 2Aδ to dephosphorylate the ChREBP protein (Iizuka and Horikawa, 2008). Long-term inhibition of ChREBP in ob/ob mice has been associated with improvements in plasma triacylglycerols (TAGs), nonesterified fatty acids (NEFAs), and insulin sensitivity restoration in both skeletal muscle and adipose tissue (Dentin et al., 2006), and therefore may someday be targeted in human chronic disease research.

3.11.2.2 Glucose and SREBP-1c

Traditionally, in the fed state, both ChREBP and SREBP-1c share lipogenic genes and genes related to the hexose monophosphate shunt. Some research groups suggest that hepatic glucokinase is required for these demonstrated synergistic effects (Iizuka and Horikawa, 2008). Although ChREBP and SREBP-1c both recognize sequences related to the E box consensus CACGTG, the binding patterns are distinct and may provide the explanation of their differential effects (Koo et al., 2001). SREBPs also belong to the family of bHLH leucine zipper transcription factors, whereby SREBP-1c is one of three isoforms and their release from the endoplasmic reticulum is followed by activation via transport to the Golgi complex a two-step cleavage process (Minihane, 2009). SREBP-1c is primarily involved in mediating insulin effects whereas ChREBP is key in mediating glucose effects (Koo et al., 2001). Insulin signaling regulates the transcription of lipogenic enzymes and is known to activate SREBP-1c (Iizuka and Horikawa, 2008). It has been shown that changing glucose concentrations has little or no effect on activation of SREBP-1c in the absence of insulin and only a modest effect in insulin's presence; glucokinase expression is dependent on insulin levels and its expression is critical for the ability of the hepatocyte to respond to elevated glucose levels (Koo et al., 2001). Authors suggest that there exists a dual level of regulation by both ChREBP and SREBP-1c to ensure that lipogenesis is not inappropriately turned on in the liver and thus only occurs when both anabolic signals from insulin and glucose metabolism are in agreement (Koo et al., 2001). SREBP-1c may prove to show some therapeutic possibility as SREBP-1c null mice show improvement in hepatic steatosis although not insulin resistance (Dentin et al., 2006).

3.11.2.3 Glucose and LXR

The LXR is part of the super family of nuclear receptors (Schuster, 2006). LXRα is most abundant in the liver but is also found in tissues related to lipid and cholesterol metabolism, whereas LXRβ is found in almost every tissue (Schuster, 2006). Both LXRs bind to the retinoid X receptor (RXR) as heterodimers to regulate gene expression. Glucose has been shown to bind directly to LXR and activate its target genes, including ChREBP and genes for cholesterol metabolism (ATP-binding cassette transporters, ABCA1 and ABCG1) in HepG2 cells which respond poorly to glucose (Denechaud et al., 2008). ChREBP has been recently identified as a target for LXR whereby LXR can directly regulate ChREBP gene expression at the transcriptional level (Denechaud et al. 2008; Iizuka and Horikawa, 2008). More recently, Denechaud et al. (2008) have found that glucose-mediated activation of ChREBP and its target genes occurred via an LXR-independent mechanism; therefore, the true relationship between LXR and ChREBP is still under investigation.

3.12.3 Cholesterol and gene interactions

Cholesterol is an essential molecule that contributes to cellular membrane fluidity and is a precursor to steroid hormones and bile acids; however, dysregulation of cholesterol metabolism can lead to atherosclerosis and increased risk of heart disease and stroke (Wang et al., 2008b). Complex regulation is needed for dietary cholesterol uptake as well as endogenous synthesis and biliary excretion of cholesterol to prevent these disease processes (Wang et al., 2006). Excess cholesterol is removed via bile, either after conversion into bile acids or as intact molecules kept in solution by bile acids and phospholipids (Wang et al., 2006).

The major bile acids in mammals are cholic acid (CA), chenodeoxycholic acid (CDCA), and muricholic acids (Wang et al., 2006). Secondary bile acids in humans that are prevalent are deoxycholic acid (DCA) and lithocholic acid, from the large intestine (Wang et al., 2006). LXR nuclear receptors are known whole-body cholesterol sensors with target genes of LXR including SREBP-1c, FAS, stearoyl-CoA desaturase-1 (SCD-1), and the ABC family of transporters (Beaven and Tontonoz, 2006). The human *CYP7A1* gene lacks an LXR response element in its promoter; therefore, it is not a direct target in humans. However, it is used regularly in rat and rabbit models (Beaven and Tontonoz, 2006).

3.12.3.1 Cholesterol and LXR/FXR

CYP7A1 catalyzes the first and rate-controlling reaction in the classic bile acid synthesis pathway (Xu et al., 2004). It has been recently shown that CYP7A1 transcription is negatively regulated by FXR and that bile acids such as lithocholic, CDCA, and DCA are ligands that activate FXR; however FXR does not directly bind to CYP7A1 but instead regulates it via activating small heterodimer protein (SHP) (Xu et al., 2004). LXR is a known positive regulator of CYP7A1, and it has been shown that the inhibitory effect of FXR overpowers any stimulatory effects of LXR, thus downregulating CYP7A1 (Xu et al., 2004). In rats, feeding cholesterol did not expand the bile acid (ligand) pool for FXR but did increase LXRα ligand oxysterols in the liver (Xu et al., 2004). FXR was not activated and there was no change in target genes SHP; however, CYP7A1 was upregulated and LXRα target gene *ABCA1* mRNA was significantly increased (Xu et al., 2004). With CA feeding instead of cholesterol, FXR was activated and decreased transcription of CYP7A1 in rats (Xu et al., 2004). The increase in plasma cholesterol with just cholesterol feeding was limited because without FXR activation, LXRα stimulated CYP7A1, and bile acid synthesis was increased, therefore absorbing cholesterol and limiting what could reach the plasma (Xu et al., 2004). CA represents the most important ligand for FXR which is dependent on SHP; under normal conditions, FXR may have most important repressor of CYP7A1—basal transcription depends more on FXR than LXR (Wang et al., 2006).

LXRα nuclear receptor has been shown to play an important and novel role in end-product inhibition of cholesterol biosynthesis (Wang et al., 2008b). Wang et al. (2008b) suggest that LXRα works with SREBP to regulate the expression of CYP51A1 in response to 25-OHC. This endogenous oxysterol, a natural potent ligand of LXR 24,25-epoxycholesterol, has been shown to be important for the acute control of cellular cholesterol homeostasis (Wang et al., 2008b). Although complete mechanisms are not fully understood, it is possible that synthetic and oxysterol binding to LXR results in different conformations of the receptor (Wang et al., 2008b). As a potential therapeutic target, Beaven and Tontonoz (2006) suggest using an LXRβ-specific drug which may increase reverse cholesterol transport through ABCA1 without increasing triglyceridemia in the liver, because LXRα controls the regulation of SREBP-1c and FAS. Activation of SREBP-1c transcription by LXR is known to induce the synthesis of oleate when sterols are in excess (Horton et al., 2002). Oleate is preferred for the synthesis of cholesteryl esters which are required for transport and storage of cholesterol (Horton et al., 2002).

3.11.3.2 Cholesterol and hepatocyte nuclear factor (HNF)-1α

Bile acid recycling takes place in the ileum involving a brush border membrane glycoprotein apical sodium-dependent bile acid transporter (*ASBT*) essential for bile acid uptake by

the ileum (Thomas et al., 2006). Basal expression of *ASBT* is highly dependent on the HNF-1α, but the direct impact of cholesterol on the *ASBT* gene is unclear (Thomas et al., 2006). Bile acid influx into Caco-2 cells is depressed by oxysterols, suggesting that *ASBT* is a sterol target gene in mice (Thomas et al., 2006). In human Caco-2 cells, chronic dietary cholesterol overload decreased ASBT mRNA and protein levels in ileal mucosa demonstrating an indirect event secondary to inhibition by bile acids (Thomas et al., 2006). The impact of SREBP-2 is proposed to be indirect, whereby only the combination of HNF-1α and active SREBP-2 synergistically transactivate the *ASBT* gene (Thomas et al., 2006). HNF-1α is not sterol sensitive; therefore, an interaction with SREBP-2 is plausible but needs more study.

3.11.4 FAs, lipids, and gene interactions

3.11.4.1 FAs and SREBP-1c

FAs are among the most intensively studied in terms of their impact on gene expression and regulation. Polyunsaturated fatty acids (PUFAs) are known to target many transcription factors, including SREBP-1c, LXR, and peroxisome proliferator-activated receptors (PPARs). PUFA can suppress lipogenic genes and induce genes involved in FA oxidation to repartition FA from TAG synthesis to oxidation (Clarke, 2004). PUFAs are known to indirectly inhibit hepatic SREBP-1c gene expression, enhancement of mRNA turnover, and interference with the proteolytic processing of the SREBP-1c protein (Clarke, 2004; Dentin et al., 2005), which favor utilization of FA for energy versus storage. Fish oils, containing n-3 PUFAs, downregulate the mature form of SREBP by decreasing SREBP-1c mRNA with a simultaneous decrease in hepatic FAS mRNA (Li et al., 2008). Reduction of nuclear SREBP-1c abundance is probably the main mechanism by which PUFA suppresses FA synthesis gene expression (Pegorier et al., 2004). N-3 PUFA suppression of *de novo* lipogenesis and monounsaturated fatty acid (MUFA) synthesis requires SREBP-1c (Jump et al., 2005).

3.11.4.2 FAs and LXR

PUFAs also downregulate lipogenesis via interaction with the LXR to regulate gene expression (Sampath and Ntambi, 2005). The relationship between PUFA regulation by SREBP and LXR cannot be ignored. It is known that SREBP-1 has two response elements for LXR, and oxysterol ligand activation of LXR induces the transcription of SREBP-1 (Clarke, 2004). PUFAs can displace oxysterols from LXR, therefore antagonizing the transactivation of LXR and may also "trap" LXR as a PPARα/LXR heterodimer (Clarke, 2004). In this way, the PUFA to oxysterol ratio is very important. When PUFAs to oxysterols is low, LXR/RXR favor lipogenic gene expression, but when the ratio of PUFAs to oxysterols is high, PPARα/LXR and PPARα/RXR favor FA oxidation (Clarke, 2004). However, there is conflicting evidence *in vitro* and *in vivo* regarding the role of LXR in PUFA gene regulation. In the review by Jump et al. (2005), the authors concluded that the LXR response element in the SREBP-1c promoter is not required for PUFA suppression of SREBP-1c gene transcription. As well, the absence of PUFA suppression on many LXR-regulated genes *in vivo* provide evidence that LXR is not involved in an *in vivo* model (Jump et al., 2005). Therefore, the true molecular involvement of direct LXR activation by PUFAs is yet to be understood.

3.11.4.3 FAs and PPARs

PPARs, α, γ, and β/δ, are also members of the super family of nuclear receptors, and their relationship with FAs is well documented (Kersten et al., 2000). PPARβ/δ is found in many tissues but in highest concentrations in the gut, heart, and kidney (Kersten et al., 2000); however, little is known about its involvement in FA sensing and therefore will not be covered in this section. The DNA-binding domain of PPARs consists of two zinc fingers and is directly related to the binding of PPAR to peroxisome proliferator response elements (PPREs) in the promoters of target genes (Cho et al., 2008). PPREs are DR-1 elements consisting of two hexanucleotides with the AGGTCA consensus sequence separated by a single nucleotide base (Cho et al., 2008). When the ligand binds to PPAR, it then binds to RXR to form a heterodimer and can recruit transcriptional coactivators (Kersten et al., 2000). PPARα is mainly expressed not only in the brown adipose tissue and liver, but also in the kidney, heart, and skeletal muscle (Kersten et al., 2000; Cho et al. 2008). It has mostly been studied in respect to its target genes of lipid catabolism such as FA uptake through membranes, FA binding in cells, FA oxidation, and lipoprotein assembly and transport (Kersten et al., 2000). PUFAs are responsible for upregulating genes via PPARα related to FA transport, metabolism, and oxidation (Sampath and Ntambi, 2005). Mostly target genes of PPARγ are directly related to lipogenic pathways, including adipocyte fatty acid-binding protein (FABP), acyl-CoA synthase, and fatty acid transport protein (FATP) (Kersten et al., 2000). In adipose tissue, PPARγ is a direct target gene of SREBP, and both of these protein levels are elevated (Kersten et al., 2000). Together they stimulate the uptake of glucose and FAs and their conversion to triglycerides for storage (Kersten et al., 2000). Under highly catabolic conditions like fasting, lipogenesis continues and is dependent on PPARγ (Kersten et al., 2000). A large area of pharmaceutical interest is the development of synthetic PPARα and PPARγ agonists, such as fibrates for serum lipid-lowering or thiazolidinediones for management of T2D, respectively. Nutrigenomics, however, can aid in using nutrients above pharmaceuticals to target many health benefits simultaneously.

3.11.5 Lipids and APOE

Genetic variation at the APOE locus is considered an important risk factor for CVD. The APOE gene encodes for apolipoprotein E, which is a protein component of TAG-rich chylomicrons and very low-density lipoprotein (VLDL) remnants (Hagberg et al., 2000). Apolipoprotein E mediates chylomicron and VLDL binding and uptake by the hepatic low-density lipoprotein (LDL) receptor, thus enhancing plasma clearance (Campos et al. 2001; Erkkila et al., 2001). The APOE gene is located on human chromosome 19, is 3.7 kb in length, and contains four exons. The most commonly studied variants of the APOE gene are three alleles E2, E3, and E4 resulting in three isoforms of the protein. These isoforms differ in amino acid sequence at positions 112 and 158 whereby E3 has a cysteine at 112 and an arginine at 158; E2 has a cysteine at both positions; E4 has an arginine at both positions (Eichner et al., 2002). APOE allele frequencies vary by ethnicity; however, E3 is the major allele in all populations (gene frequency of 0.49–0.91), then followed by E4 (gene frequency 0.06–0.37) and E2 (gene frequency 0–0.15) (Campos et al., 2001). These missense mutations in the APOE gene locus are associated with varying serum LDL-cholesterol concentrations (Corella et al., 2001), whereby plasma total cholesterol and LDL-cholesterol concentrations are lowest in subjects with the E2 allele, intermediate in

those with the E3 allele, and highest in those with the E4 allele (Erkkila et al., 2001). Furthermore, the E4 allele is associated with a greater risk of coronary artery disease possibly stemming from known increased cholesterol absorption, increased LDL production from VLDL, decreased bile acid synthesis, and LDL clearance from plasma with this genotype (Lahoz et al., 2001).

3.11.6 Diet and APOE

Lahoz et al. (2001) demonstrated that among participants of the Framingham Study, male carriers of both E2 and E4 alleles were at increased risk of CVD. The E2 compared to the E3 variant, despite an association with lower LDL-cholesterol, actually binds more poorly to lipoprotein receptors leading to delayed clearance and increased accumulation of plasma chylomicron and VLDL remnants (Campos et al., 2001). In the study by Campos et al. (2001), E2 carriers with intakes of higher saturated fat were associated with increased VLDL-cholesterol, decreased HDL-cholesterol, and smaller LDL particles, whereas the opposite or no effect was found in the E4 and E3 carriers. Similarly, the E4 allele is associated with higher LDL-cholesterol and triglyceride and lower HDL-cholesterol concentrations, with diets high in saturated fat and cholesterol (Campos et al., 2001). Interestingly, male E4 carriers may be the most responsive to a cholesterol-lowering diet (Lopez-Miranda et al., 1994). The authors showed that male E4 carriers had significantly more serum LDL-cholesterol reduction than other participants with a diet meeting the National Cholesterol Education Program (NCEP) Step I criteria (Lopez-Miranda et al., 1994).

Studies on the relationship between APOE polymorphisms and carbohydrate intake are rare; however, some are worth mentioning. Erkkila et al. (2001) investigated patients with coronary artery disease for the interaction between APOE genotype and dietary intakes of fat and sucrose on serum lipids. Results showed high serum TAG concentration in E2 carriers with a high sucrose intake, while high serum TAG concentration was observed in E4 carriers with a low sucrose intake (Erkkila et al., 2001). In a 2-week chronic intervention study, Jenkins et al. (1993) found that E2 carriers versus noncarriers were more responsive to a dietary intervention of increased dietary fiber. Also, the effect of oat bran resulted in LDL-cholesterol lowering, which was not seen with the wheat bran intervention (Jenkins et al., 1993). More recently, in a 6-week chronic intervention, Tucker et al. (2010) showed that fasting serum lipid response to consumption of whole grain wheat sourdough bread in healthy adults of low and high CVD risk depended on the presence of the APOE E3/E3 genotype. Specifically, the control white bread demonstrated reduced fasting LDL-cholesterol in normoglycemic/normoinsulinemic adults, and reduced fasting TAG and TAG: HDL-cholesterol in hyperglycemic/hyperinsulinemic adults with the APOE E3/E3 genotype (Tucker et al., 2010).

3.11.7 Lipids and hepatic lipase (HL)

HL activity is also another risk factor for CVD, playing a critical role in lipid metabolism, particularly related to HDL particles (Connelly, 1999). HL is located on human chromosome 15q21 (van't Hooft et al., 2000), and its primary action is to hydrolyze triglycerides and phospholipids in plasma lipoproteins on the sinusoidal endothelial surface (Connelly, 1999). Increased HL activity is associated with small LDLs and low HDL because HL can increase the hepatic uptake of HDL lipids (Grundy et al., 1999). There are several

known polymorphisms in the 5′ flanking region of HL gene promoter (LIPC), which are in complete linkage disequilibrium (Couture et al., 2000). Couture et al. (2000) demonstrated that the rare allele mutation of these polymorphisms, such as LIPC −514C>T polymorphism which produces a C>T substitution at nucleotide 514, is considered favorable, whereby T carriers participating in the Framingham Study had decreased HL activity and higher HDL-cholesterol concentrations.

3.11.8 Diet and LIPC

Ordovas et al. (2002) also used subjects from the Framingham Study to show that −514T allele carriers had significantly greater HDL-cholesterol concentrations when consuming less than 30% of energy from fat; however, mean HDL-cholesterol concentrations were lowest among TT individuals if the total intake of fat was greater than 30% of energy and there were no differences observed between CT and CC individuals (Ordovas et al., 2002). These results were confirmed in more recent work by Tai et al. (2003) who showed that TT participants had higher serum TAG levels when consuming greater than 30% of energy from fat. Studies of the common −250G>A polymorphism have also shown favorable effects on lipid metabolism. For example, in a 3-month chronic intervention conducted by Lindi et al. (Lindi et al., 2008) the rare −250A allele was associated with low HL activity, and participants with the AA genotype responded favorably to a high MUFA diet, with an observed greater reduction in serum LDL-cholesterol compared to other genotypes. Carbohydrate feeding studies that measure LIPC polymorphisms are rare; however, in the aforementioned 6-week chronic bread intervention by Tucker et al. (2010), there were no observed effects of LIPC genotype on fasting serum lipid response to whole grain wheat sourdough bread compared to white bread in individuals of low and high risk for CVD.

3.11.9 Interaction between APOE and HL

Furthermore, recent work by Wood et al. (2008) demonstrated a clear gene–gene interaction between APOE and LIPC gene loci. The effects of such interaction influenced plasma TAG concentrations, likely due to their combined role pertaining to the uptake and clearance of TAG-rich lipoproteins. In summary, young Canadian participants with the APOE E2 genotype had significant variations in plasma TAG levels between LIPC −514CC (0.87 ± 0.27) versus LIPC −514TT (1.32 ± 0.34) genotypes. Similarly, participants with the APOE E2 genotype demonstrated significant variations in TAG levels between LIPC −250GG (0.87 ± 0.27) and LIPC −250AA (1.29 ± 0.32) genotypes (Wood et al., 2008). This work presents fundamental knowledge regarding optimal APOE and LIPC polymorphisms to protect against hypertriglyceridemia in a healthy population and reinforces the need to study potential gene–gene interactions in future population and clinical studies.

3.12 VITAMIN A AND GENE INTERACTIONS

Vitamins A, E, and D provide classic examples of how nutrients modulate gene expression. Vitamin A or retinol is an essential dietary component that can act as a hormone and regulate gene transcription. Classic functions of this vitamin include a role as a cofactor in

vision, and acting as a hormone in reproduction, embryonic development, bone growth, immune response, growth and differentiation of epithelia, and inhibition of tumor growth (Wolf, 2008). Vitamin A in its active form, all-trans retinoic acid (RA), can bind to nuclear receptors retinoic acid receptor (RAR) and RXR (Cai and Gudas, 2009). RXRs form heterodimers with various nuclear receptors, such as the estrogen receptor (ER), vitamin D receptor (VDR), thyroid hormone receptor (TR), PPAR, FXR, LXR, constitutive androstane receptor (CAR), and pregnane X receptor (PXR), some of which have been discussed in this chapter (Wang et al., 2008a). Within the families of nuclear receptors RAR and RXR, there exists a highly conserved DNA binding domain which recognizes and allows the binding of sequence-specific DNA known as retinoic acid response elements (RAREs) (Bastien and Rochette-Egly, 2004). Without ligands present, retinoid receptors are found mostly in the nucleus, and in order to activate gene expression, ligand-induced conformational changes need to occur to cause the dissociation of corepressors and recruitment of coactivators (Bastien and Rochette-Egly, 2004).

3.12.1 Dual roles of vitamin A

Interestingly, RA can provide cellular signaling for two opposing responses depending on the cytosolic concentrations of related binding proteins. To elicit growth inhibition in MCF carcinoma cells, cytosolic RA-binding protein transports RA into the nucleus to attache to RAR, whereby the complex dissociates and frees RA-RAR which can then bind to a desired gene (Wolf, 2008). Conversely, for cell proliferation in keratinocytes, RA is channeled into the nucleus by FA-binding protein 5, whereby it can bind to and activate PPARβ/δ (Wolf, 2008); this dual role shows tissue-specific actions which are overall beneficial to health.

3.13 VITAMIN E AND NUTRIGENOMICS

The lipid-soluble vitamin E has many physiological roles, many of which were only recently discovered and will require more investigation. Vitamin E is absorbed directly through the intestine, and is metabolized to any of eight different antioxidant compounds (Schneider, 2005). There are four tocopherols (α-, β-, γ-, and δ-), and four tocotrienols (α-, β-, γ-, and δ-), although α-tocopherol is known to be the most chemically and biologically active of all isoforms (Schneider, 2005). The other isoforms of vitamin E are poorly studied and are therefore poorly understood physiologically; however, it is possible that some of the nontraditional roles of vitamin E recently developed may be attributed to more than just α-tocopherol.

3.13.1 Vitamin E and atherosclerosis

Vitamin E is a potent antioxidant compound as well as an important signaling molecule. It is understood that vitamin E is involved in the lipid-peroxidation pathway and protects against free radicals (Azzi et al. 2004; Schneider, 2005). As a signaling molecule, vitamin E may also be involved in four events of early atherosclerosis, including the inhibition of monocyte-endothelial cell adhesion, platelet adhesion and aggregation, formation of COX-2 and 5-LOX and of scavenger receptors SR-A and CD36 (Schneider, 2005). Vitamin

E in the form of tocotrienols in palm oil also has been shown recently to activate PPARs and attenuate development of atherosclerosis in Apo E-deficient mice through target of LXRα (Li et al., 2010); therefore, Vitamin E could be important in modulating early risk of CVD.

3.13.2 Vitamin E and cholesterol biosynthesis

Recent work by Valastyan et al. (2008) has demonstrated a novel role for vitamin E in cholesterol biosynthesis. It is well-known that 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG)-CoA reductase is the rate-limiting enzyme of *de novo* synthesis of cholesterol and that SREBP-2 is responsible for its upregulation. The authors showed that genomic responses to vitamin E are mediated via SREBP-2 and that α-tocopherol appears to attenuate the proteolytic activation of SREBP-2 and its translocation into the nucleus, suggesting that α-tocohpherol may share the same sensing mechanism as sterols do for the SREBP-2 transcription factor in the sterol sensor SREBP cleavage-activating protein (SCAP) (Valastyan et al., 2008). Vitamin E's influence of SREBP-2 and cholesterol biosynthesis are not clearly understood; however, this work provides an interesting novel mechanism of α-tocopherol as a nonantioxidant.

3.14 VITAMIN D AND GENE INTERACTIONS

Vitamin D is an important contributor to the endocrine system, with unique roles in many target organs. Primarily, vitamin D acts on bone, intestine, kidney, and liver, and may be involved in the etiology of many diseases, including osteroporosis, cancer, diabetes, and CVD (Valdivielso and Fernandez, 2006). The activated form, 1,25(OH)2D3, is able to function as the ligand for the VDR, which consists of a dual zinc finger-based DNA-binding domain, nuclear localization signal, and a ligand-binding domain (Jurutka et al., 2007). Once VDR is activated, VDR heterodimerizes with RXR, and this complex can bind to vitamin D response elements (VDREs) and initiate the recruitment of other nuclear proteins to form the transcriptional preinitiation complex (Valdivielso and Fernandez, 2006; Jurutka et al., 2007). The activated complex recruits another coactivator complex called the vitamin D receptor-interacting protein complex, and acetylated histones help relax the chromatin structure in order to make DNA more accessible and allow for gene transcription (Gocek et al., 2007; Jurutka et al., 2007). 1,25-Dihydroxyvitamin D3 can activate intracellular signaling pathways important to T2D and CVD research such as protein kinase C, mitogen-activated protein kinases, and phosphatidylinositol 3-kinase (Gocek et al. 2007; Jurutka et al., 2007).

3.14.1 Vitamin D and breast cancer

VDR and breast cancer is an interesting area of research due to potential downregulation of VDR function leading to loss of sensitivity to *1,25-dihydroxyvitamin D*3 in many transformed breast cancer cells (Mittal et al., 2008). VDR also has involvement in modulating proteins for cell signaling, proliferation, differentiation, and survival of normal mammary epithelial cells (Mittal et al., 2008); therefore, VDR may play a critical role in the transition between healthy and malignant cells.

3.14.2 Vitamin D and FAs

VDR induces calcemic and phosphatemic effects to maintain normal bone mineralization and remodeling (Jurutka et al., 2007). Interestingly, it is also able to act as a sensor for detoxification in some tissues such as the colon, where it can bind to secondary bile acid lithocholate. Novel findings by Jurutka et al. (2007) indicate that PUFA and curcumin can bind directly to VDR and induce RXR recruitment and activate transcription via a VDRE. These results support the beneficial biological effects that PUFA may exert on bone health as well as in colon and skin (Jurutka et al., 2007). The potential of VDR to act as a sensor for circulating FAs such as PUFA and other dietary lipids such as curcumin poses exciting new areas of dietary interactions to consider in future research.

3.15 PHYTOESTROGENS AND GENE INTERACTIONS

3.15.1 Phytoestrogens and breast cancer

There is also a growing interest in the effects on gene expression with regard to dietary intake of phytochemicals, such as phytoestrogens, phytosterols, and polyphenols. Phytoestrogens are made of three main classes, isoflavones, coumestans, and lignans, all of which are diphenolic compounds similar to estrogen and are able to bind to the ER (Kris-Etherton et al., 2002). There are three main classes of phytoestrogens: isoflavones, coumestans, and lignans. In particular, soy isoflavones, such as genistein and daidzein are associated with lower incidence of breast and prostate cancers (Dip et al., 2008). Most estrogenic responses are mediated by ERα and ERβ, both of which associate with coregulatory partners to remodel chromatin and regulate transcription of downstream genes and bind to the same consensus estrogen-responsive element, but they show partially antagonistic effects (Dip et al., 2008). 17β-Estradiol does not discriminate between ERα and ERβ but phytoestrogens bind to ERβ with five times higher affinity than ERα (Dip et al., 2008). Like other ER agonists, phytoestrogens stimulate proliferation of estrogen-sensitive tumor cells but may have a biphasic effect during cancer depending on time of exposure (Dip et al., 2008). In late-stage cancer cells, phytoestrogens may induce a transcriptional profile that promotes proliferation of cells expressing high levels of ERα and little ERβ (Dip et al., 2008).

3.15.2 Phytoestrogens and lipid, glucose metabolism

Many studies that recently examine the ability of phytoestrogens to bind to nuclear receptors to regulate gene expression involve coumestrol. Recent work by Takahashi et al. (2008) demonstrated that coumestrol increased FXR transcriptional activity and SHP-promoter containing reporter gene in a dose-dependent manner. FXR activation induces expression of SHP which acts as a corepressor for several nuclear receptors including LXRα and HNF-4α (Takahashi et al., 2008). HNF-4α is involved in lipid transport and glucose metabolism through the FXR-SHP cascade. Although coumestrol also binds to both ERα and ERβ to lower serum total cholesterol and prevent ovariectomy-induced bone loss, HepG2 cells lack any ER activities, suggesting that coumestrol is able to increase SHP gene expression through direct activation of FXR (Takahashi et al., 2008). Overall coumestrol may act through ER-mediated and FXR-mediated pathways to influence lipid and glucose metabolism, but further investigation is needed.

3.16 PHYTOSTEROLS AND GENE INTERACTIONS

Phytosterols and their saturated forms, phytostanols, are present in the nonsaponifiable fraction of plant oils (Kris-Etherton et al., 2002). In the diet, typically consumed forms include sitosterol, stigmasterol, and campesterol, which are not made in humans and are poorly absorbed and stored (Kris-Etherton et al., 2002).

3.16.1 Phytosterols and cholesterol metabolism

Although mechanisms are not greatly understood, consumption of phytosterols with or without simultaneous ingestion of dietary cholesterol can reduce serum cholesterol concentrations (Plat et al., 2005), which may be explained by their interaction with LXR (Calpe-Berdiel et al., 2007). Plat et al. (2005) found that plant sterols clearly could activate LXR in Caco-2 cells and that the change of ABCA1 was proportional to the change in LXR-activating potential in these cells. LXR agonists are known to have a regulatory role in cholesterol metabolism and hepatic expression of genes in FA metabolism (Plat et al., 2005). Therefore, it is tempting to suspect that indeed it is LXR activation accounting for the decrease in serum cholesterol after phytosterol feeding. However, more work needs to be done across models.

3.16.2 Phytosterols and cancer

In addition to this great potential for protection of CVD through serum lipid lowering, phytosterol compounds may also protect against common Western cancers. Phytosterols have effects on tumor and host tissue membrane structure and function, signal transduction and immune function on breast, prostate and colon cancers (Awad and Fink, 2000). Woyengo et al. (2009) recently published an excellent review regarding the role that phytosterols may play in the prevention of cancer and therefore will not be covered in depth here.

3.17 POLYPHENOLS AND GENE INTERACTIONS

Phenolic compounds, also known as polyphenols, are present in all plants with more than 8000 different phenolic structures (Kris-Etherton et al., 2002). Serum polyphenol levels depend on the type, quantity, and quality of plant foods in the diet, estimates which are hard to derive because many phenolic compounds are missing from databases (Kris-Etherton et al., 2002).

3.17.1 Polyphenols and CVD

Despite lacking dietary intake information, an inverse relationship exists between flavonoid intake and risk for coronary disease and cancer (Kris-Etherton et al., 2002). In relation to CVD, polyphenols exert anti-inflammatory activity by modulation of proinflammatory gene expression such as cyclooxygenase, lipooxygenase, nitric oxide synthase (NOS), and other cytokines working through nuclear factor-kappa B, mitogen-activated protein kinase signaling, and through activation of PPARγ (Santangelo et al., 2007). Furthermore,

polyphenols modulate the nitric oxide synthase family, inhibiting nitric oxide release by suppressing NOS enzymes, making them desirable anti-inflammatory compounds (Santangelo et al., 2007). Increased eNOS expression may help to ameliorate endothelial dysfunction, harmonize blood pressure, and prevent atherosclerosis as a long-term beneficial effect (Santangelo et al., 2007). Polyphenols also have antiangiogenic and antiproliferative activity which inhibit inflammatory mediators and downregulate the expression of transcription factors and genes involved in hypertension (Nicholson et al., 2008). Therefore, it is clear that polyphenols have potential in modifying CVD risk via additive and/or synergistic mechanisms (Chong et al., 2010).

3.17.2 Polyphenols and cancer

As well, many polyphenols have been recognized as having anticarcinogenic effects. Although anticarcinogenic effects are concentration dependent, apoptosis induction may be the most important target (Ramos, 2008). For example, supplemented lycopene decreases tumor growth in human cancer cell lines by interrupting the PI3K/Akt phosphorylation activation pathway thereby reducing tumor angiogenesis and progression of tumor development (Tang et al., 2008). Phytochemical research is promising; however, there is increasing evidence that consumption of whole foods is better than isolated compounds to obtain synergistic benefits (Ovesna et al., 2008). It remains challenging to fully assess the human health impact of such compounds because of the diversity where composition and bioavailability may be unknown.

3.18 NUTRIGENOMICS SUMMARY: ADVANTAGES, LIMITATIONS, FUTURE

Scientists are only at the frontier of understanding the complex mechanisms and interactions between dietary factors, transcription factors, and their respective target genes. Advantages of nutrigenomics include the basic level research which allows for manipulation of nutrient exposures and target genes made possible in a variety of cell lines, tissues, and animal models. The development and use of microarray technology greatly increases the scope to which gene expression can be measured and genome-wide assays can help narrow the vast number of potential pertinent genes to study. Ultimately, nutrigenomics may guide development of novel foods for improving chronic disease risk and management. However, there are also challenges that deserve mention. The search for appropriate biomarkers in chronic disease risk continues for multifactorial diseases. Most foods are made up of many known or unknown bioactive compounds, and the potential of unknown gene–gene interactions is significant.

To address these limitations, there are areas of nutrigenomic research that could be improved. One of the most dynamic is the need for better communication with databases to inventory, assimilate and disseminate results that can shape future research. Consistency and experimentation in physiologically relevant tissues or cell lines is also important for repeatability and validity. Finally, perhaps the largest concern is addressing the complex relationship of nutrients and other bioactive compounds within a food matrix, which will undoubtedly improve with dynamic research. The ever-evolving field of nutrigenomics, albeit in its infancy, has the potential to modernize prevention and management of chronic diseases through human diet.

3.19 CONCLUSIONS

Post-genomic era, the research fields of nutritional genomics are rapidly advancing. Such progressions will undoubtedly lead to a superior understanding of the effects of the human diet on chronic disease prevention and treatment. The genome, epigenome, and transcriptome are intrinsically connected and thus, dietary nutrients, non-nutritive compounds, and their synergistic actions have potential in modifying all three fields of research simultaneously. Thus, both targeted, hypothesis-driven and multidisciplinary efforts will be necessary to help realize the ultimate goal of efficacious preventative and therapeutic treatments for human chronic diseases. Phytochemicals appear to have a unique role in nutritional genomics and may be at the therapeutic forefront.

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4 Nutraceuticals and Antioxidant Function

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4.1 INTRODUCTION

The role of oxidative stress in initiating and perpetuating major human diseases necessitates an understanding of endogenous and exogenous reactive oxygen species (ROS) and antioxidant functions. In many chronic conditions, oxidants overwhelm the antioxidant capacity of the cells, resulting in tissue damage and inflammation. In theory, supplementation with nutraceuticals from plant and animal sources can introduce antioxidants into the body and stave off the deleterious effects of oxidation. The goals of this chapter are to (1) provide an overview of oxidative stress as it pertains to disease; (2) introduce phytochemicals, amino acids, peptides, and proteins with antioxidant and antioxidative stress properties; (3) assess recent developments in the measurement of antioxidant capacity and oxidative damage; and (4) present evidence of antioxidant health benefits.

4.2 OXIDATIVE STRESS AND ROS

Oxidative stress is imposed on the body's cells when the level of ROS outweighs the reducing capacity of antioxidant and antioxidative stress mechanisms. While a low level of stress is always present, an increase in the amplitude and duration of stress can result in damage to cell membranes, proteins, and DNA. This in turn can set the conditions for a new disease or can exacerbate an existing condition. Inflammatory bowel disease, ischemia/reperfusion disorders, and cancer have all been linked to oxidative stress (Aw, 1999; Rezaie et al., 2007; Roessner et al., 2008) and in fact, biomarkers of oxidative damage in diseased tissues are often disproportionately altered and in most cases, are higher than that present in healthy cells (Siegers et al., 1984; Rezaie et al., 2007).

ROS encompass molecules with varying degrees of reactivity which can be free radicals or nonradicals, but they all are associated with oxygen. Common ROS formed within biological systems include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and hypochlorous acid (HOCl). Superoxide anions exhibit relatively low reactivity to other molecules and upon formation rapidly dismutate to H_2O_2 at physiological

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pH (Halliwell and Gutteridge, 2007d). Within the cell, endogenous superoxide dismutase (SOD) enzymes execute a similar function. Although H_2O_2 is one of the least reactive ROS, like water, exogenously produced H_2O_2 can cross cellular membranes by utilizing aquaporin channels to gain access to internal milieu (Bienert et al., 2007). As a result, H_2O_2 can disrupt redox-sensitive signaling molecules such as kinases and transcription factors such as nuclear factor kappa-B (NFκB) and activator protein-1 (AP-1), thereby effecting a change in cell signaling and gene expression (Halliwell and Gutteridge, 2007b). In Caco-2 human intestinal adenocarcinoma cells, which are often used to assess antioxidative stress properties of nutraceuticals, the addition of H_2O_2 results in the increased secretion of interleukin (IL)-8, a proinflammatory cytokine under the transcriptional control of NFκB (Yamamoto et al., 2003).

Both the hydroxyl radical and hypochlorous acid are highly reactive *in vivo* and can indiscriminately attack surrounding cell components. While ·OH damage is limited to the immediate vicinity, HOCl can also cross membranes and oxidize lipids, proteins, and DNA, thereby amplifying the damage potential (Halliwell and Gutteridge, 2007d). The relative importance of damage to these different molecular targets in mediating cell injury or death depends upon the degree of oxidative stress, by what mechanism it is imposed, for how long, and the nature of the system stressed (Droge, 2002). ROS, especially ·OH radicals, are mutagenic and participate in a range of DNA alternations including DNA cleavage, DNA-protein cross-linking, and purine oxidation (Halliwell and Gutteridge, 2007b). If the DNA repair systems are not able to fix or regenerate intact DNA, a mutation will result from the erroneous base pairing during replication, and these mutations have been linked to cancer (Marnett, 2000; Nordberg and Arner, 2001). Lipid oxidation can lead to membrane damage (Halliwell et al., 2000a) while oxidant reactions with amino acid residues in proteins result in damage ranging from modified and less active enzymes to denatured and nonfunctioning proteins (Stadtman and Berlett, 1998).

4.2.1 Endogenous sources of ROS

In eukaryotes, superoxide anion is primarily produced: when electrons leaking from the mitochondrial electron transport chain reduce oxygen, during tissue injury by xanthine oxidase, via auto-oxidation reactions in the presence of transition metal ions, during cytochrome P450 cycling, and at inflammatory sites by activated neutrophils and phagocytes via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Halliwell and Gutteridge, 2007d). Under physiological conditions, it is estimated that of the 90% of inhaled oxygen consumed by the mitochondria, about 1–3% of the oxygen molecules are converted into superoxide (Brookes et al., 2002) with H_2O_2 formed almost immediately following $O_2^{\bullet-}$ production.

H_2O_2 can react with Fe^{2+} or Cu^{+} via the Fenton reaction to form extremely damaging ·OH (Imlay et al., 1988). During inflammation, the enzyme myeloperoxidase (MPO) in phagocytes catalyzes the formation of HOCl from H_2O_2 or ·OH (Proctor, 1996). Activated phagocytes are also induced to produce high sustained levels of nitric oxide (NO) (Mayer and Hemmens, 1997; Carr et al., 2000) which react with superoxide anion to produce the powerful reactive nitrogen species, peroxynitrite ($OONO^{-}$). $OONO^{-}$ can directly oxidize lipoproteins (Heinecke, 1998) or give rise to other species that promote oxidation (Eiserich et al., 1998; Radi et al., 2001). The formation of strongly oxidizing agents is crucial for the elimination of foreign agents during inflammation. Unfortunately during this "oxidative

burst," ROS are nondiscriminant, and host tissues are similarly attacked. The inability of the body's immune system to control and downregulate ROS and inflammation is a common feature of chronic inflammatory conditions.

4.2.2 Exogenous sources of ROS

Exposure to ultraviolet (UV) radiation (Herrling et al., 2003), overexercise (Vina et al., 2000), and extrinsic xenobiotics found in tobacco smoke (Valavanidis et al., 2009), antitumor agents (Lou et al., 2006; Outomuro et al., 2007), heavy metals (Leonard et al., 2004), and organic pesticides (Drechsel and Patel, 2008; Somayajulu-Nitu et al., 2009) increase the ROS burden in the body. Oxidative stress occurs at the sites of exposure which can include the skin, lungs, gastrointestinal tract, and specialized detoxification organs like the liver and kidney. In addition to these xenobiotics, the ingestion of certain foods and drinks are associated with an increase in oxidative stress (Halliwell et al., 2000b). Compounds present in foods such as transition metal ions, heme from meats, isoprostanes, additives, lipids, and their oxidized products act as oxidants, exerting their biological effects on the stomach, small intestines, and large intestines. Lipid hydroperoxides in particular are potentially toxic products of peroxidized polyunsaturated fatty acids (PUFAs) derived from dietary fats (Sevanian and Hochstein, 1985; Girotti et al., 1987). These natural mutagens and carcinogens can initiate degenerative processes through the generation of oxygen radicals, which may ultimately lead to disorders of the digestive system (Parks et al., 1983) such as intestinal inflammation and cancer (Ames, 1984).

4.3 ANTIOXIDANTS AND ANTIOXIDATIVE DEFENSE SYSTEMS

An antioxidant is a substance that impedes or averts the development of oxidation, or helps repair or remove the oxidative damaged target (Halliwell and Gutteridge, 2007a). In this manner antioxidants are capable of affecting oxidation at all stages and may be able to forestall the development of disease. There is not one antioxidant that can accomplish all tasks; however, harnessing the effects of dietary antioxidants and enhancing endogenous antioxidant and antioxidative defenses may bring us closer to a combined strategy toward oxidation and oxidative consequences.

4.3.1 Endogenous antioxidants and antioxidative defenses

4.3.1.1 Glutathione (GSH)

GSH is tripeptide of γ-glutamylcysteinylglycine which is ubiquitously present in millimolar concentrations in all cell types (Meister and Anderson, 1983). GSH can directly scavenge free radicals or act as a substrate for glutathione peroxidase (GPx) and glutathione *S*-transferase (GST) during detoxification of H_2O_2, lipid hydroperoxides, and electrophilic compounds. Normal cellular GSH concentration is maintained through *de novo* synthesis from sulfur-containing precursor amino acids (cysteine and methionine), regeneration from glutathione disulfide (GSSG), and GSH uptake from exogenous sources via Na^+-dependent transport systems (Shan et al., 1990).

De novo GSH synthesis is catalyzed by two sequential adenosine-5′-triphosphate (ATP)-dependent reactions involving γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (Griffith, 1999). The availability of cysteine and the concentration of GSH already present in the cell will limit the activity of γ-GCS (Lu, 2009). During oxidative stress conditions, GSH is oxidized to a disulfide form, GSSG. GSSG is normally present at less than 1% of total GSH; however, this concentration is elevated during severe oxidative stress (Moylan and Reid, 2007). Once the cell senses the GSH:GSSG thiol redox imbalance, oxidant response transcriptional elements become activated. This leads to the upregulation of γ-GCS and antioxidative stress genes.

GSH present in foods and secreted in the bile can contribute to GSH concentrations in the intestinal lumen. In the small intestine, luminal GSH has been shown to be transported intact into mucosal cells (Iantomasi et al., 1997; Aw, 1999). GSH can also be cleaved into constituent amino acids (Glu, Cys, and Gly) by γ-glutamyltranspeptidase and dipeptidase and then absorbed and resynthesized to GSH once inside the cell (Iantomasi et al., 1997).

4.3.1.2 Antioxidative stress enzymes

There are a host of antioxidative stress enzymes that also tightly control unwanted ROS accumulation. SOD catalyzes the dismutation of superoxide anion to H_2O_2. Mn-SOD is located in the mitochondria while Cu- and Zn-SODs are present in the cytosol (Rodriguez et al., 2000). Once H_2O_2 is formed, it can then be converted into water by the enzymes catalase (CAT) or GPx. The gastrointestinal isoform of glutathione peroxidase (GI-GPx or GPx2) not only scavenges H_2O_2 but also alkyl hydroperoxides from absorbed lipids and protects against intestinal damage, inflammation, and malignancies (Brigelius-Flohe, 1999; Wingler et al., 2000). GPx and glutathione reductase (GR) function as a pair in the reduction of peroxides with the concomitant oxidation of GSH to GSSG and the regeneration of GSH with reducing equivalents donated from NADPH (Halliwell, 1990; Cimino et al., 1997). Although these antioxidant enzymes are synthesized and found within cells, some of the SOD and GPx activities in the intestine and colon appear to be located extracellularly, at the external cell surface (Frederiks and Bosch, 1997; Tham et al., 1998), and is believed to detoxify and prevent reactive species from reaching the gut surface.

Under conditions of oxidative or electrophilic stress, the genes encoding antioxidant and detoxification enzymes are controlled by the Nrf2-ARE (nuclear factor-E2-related factor 2–antioxidant response element) signaling pathway (Nguyen et al., 2009). During oxidative stress, the low GSH:GSSG disrupts the thiol redox status of the cell and triggers the transcription of AREs. AREs are found in the promoter region of antioxidant enzymes including SOD, CAT, γ-GCS, GST, GR, and GPx (Dhakshinamoorthy et al., 2000). ARE sequences are strictly regulated by transcription factors such as Nrf2 which, upon binding, positively regulates activity (Lee and Johnson, 2004; Nguyen et al., 2009). In a nonstressed cell, Nrf2 is bound to the Kelch-like ECH-associated protein 1 (Keap1) in the cytosol (Itoh et al., 2003). In response to oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus. Upon nuclear translocation, Nrf2 binds to ARE in the promoter region of antioxidant enzymes (Wasserman and Fahl, 1997), to *trans*-acting factors such as Maf-F/G/K (Dhakshinamoorthy et al., 2000), and to coactivators of ARE such as cAMP response element binding protein (CREB)-binding protein (CBP)/p300 (Shen et al., 2004), which coordinately regulate the ARE-driven antioxidant gene transcription.

4.3.1.3 Thioredoxin systems

Thioredoxin also contributes significantly to antioxidant defense and redox regulation. Thioredoxin acts as a reducing agent for ribonucleotide reductase and methionine sulfoxide reductases which repair oxidative damage to methionine residues in proteins (Halliwell and Gutteridge, 2007a). Reduced thioredoxin contain two thiol groups which form a disulfide in oxidized thioredoxin. Similar to the role of GR and GSSG, thioredoxin reductase reduces oxidized thioredoxins by using NADPH as an electron donor (Nordberg and Arner, 2001). Peroxiredoxin coupled with thioredoxin reduces H_2O_2 and organic peroxides, and are believed to be more important than CAT and GPx in removing peroxide in mammalian cells (Halliwell and Gutteridge, 2007a).

4.3.1.4 Additional antioxidants

Other endogenously synthesized antioxidants present in the body include uric acid, bilirubin-bound albumin, and albumin itself (Ames et al., 1981; Hulea et al., 1995). Histidine-containing peptides such as carnosine, anserine, and balenine exhibit strong antioxidant properties in muscle (Boldyrev and Johnson, 2002; Kim et al., 2002). His-Pro (commonly known as cyclo) also enhances cellular antioxidant capacity (Minelli et al., 2008). Melatonin too has been shown to protect cells against oxidative damage (Ghosh et al., 2006). Amino acids, peptides, and even proteins can act as antioxidants since they exhibit low, specific antioxidant activity (Droge, 2002).

4.3.2 Dietary antioxidants

Although endogenous antioxidants and antioxidative stress enzymes are effective in the removal of ROS, the added burden of environmental ROS and the reduction of plasma and cellular antioxidant potential as we grow older (Rizvi et al., 2006), stretches our antioxidant defenses to the limit. One further complication of old age is our reduced ability to absorb biomaterial including antioxidants (Elmadfa and Meyer, 2008) which increases our risk of oxidative damage and disease. Nutrition and dietary patterns have a direct impact on the health of the population and of selected patient groups (Caballero, 2003). Individuals in the Mediterranean area have a lower risk of several chronic diseases including colon cancer, and the use of large amounts of vegetables and fruits, cooked tomatoes, and olive oil is believed to bring about this lowered risk (Weisburger, 2002). Antioxidants found in food are predominantly free radical scavengers that directly neutralize free radicals, reduce peroxide concentrations and repair oxidized membranes, and quench iron to decrease ROS production (Parke, 1999).

Phytochemicals, amino acids, and peptides and proteins form a large part of our diet. By isolating and studying these specific components, we can understand how they interact with other biomolecules to reduce oxidative stress.

4.4 PHYTOCHEMICALS

Phytochemicals with antioxidant activities can be found in many plants, particularly in foods, spices, and medicinal herbs. Thousands of these natural products have been identified to belong to many structurally unique chemical families. While there have been

different ways of categorizing these naturally occurring compounds, source of origin, biological function, and chemical structure are among the most often used methods. Table 4.1 lists the major groups of antioxidative phytochemicals and their representative compounds found in commonly consumed fruits, vegetables, and grains according to their chemical structures (Tsao and Deng, 2004; Tsao et al., 2007; Tsao and McCallum, 2009).

4.4.1 Polyphenols

The term "polyphenols" is a highly inclusive, and sometimes, a very loose one that covers many different subgroups of phenolic acids and flavonoids. This is perhaps the largest group of phytochemicals found in food, with more than 5000 polyphenols and over 2000 flavonoids having been identified (Harborne, 1994). Polyphenols come with different structural features: benzoic acids and cinnamic acids are relatively simple, having generally a single-ring structure; however, flavonoids are more complicated, and even though they share the same backbone, they can be further classified into many subgroups such as anthocyanins, flavan-3-ols, flavones, flavanones and flavonols, and more. Some of the flavonoid subgroups such as the flavan-3-ols can be found as dimers, trimers, and polymers. Polyphenols, like other phytochemicals, are produced by plants as a chemical defense against invading insects or diseases, and to avoid phytotoxicity against themselves, these compounds are normally stored in cells as glycosides. Some flavonoids are also associated with acylated glycosides. This further complicates the phenolic profiles of plants (Beecher, 2003).

4.4.1.1 Phenolic acids

The overwhelming majority of the phenolic acids in food plants are derivatives of either benzoic acid or cinnamic acid. These phenolic acids can be found in free forms in fruits, vegetables, and grains; however, in grains or oil seeds, particularly in the bran or seed coat, many of these compounds are bound and thus are only available by acid or alkaline hydrolysis or enzyme digestion. Benzoic acids such as gallic acid are often associated with sugar molecules through ester bonding. These are often referred to as hydrolyzable tannins. Figure 4.1 lists some common phenolic acids in food.

4.4.1.2 Flavonoids

Flavonoids are ubiquitous and the most diverse in land plants. Some of the most important antioxidative and bioactive compounds have been identified in this group of polyphenols. Most flavonoids share the same flavonoid backbone structure (Fig. 4.2), which consists of two benzene rings (rings A and B) linked by an oxygen-containing heterocycle also known as chromane ring (ring C, Fig. 4.2). For most flavonoids, the B ring is attached to position 2 of the C ring; however, it can also be attached to positions 3 (isoflavones) or 4 (neoflavonoids) of the C ring. Chalcones (Fig. 4.2), despite lacking the heterocyclic ring C, are still often considered by many authorities to be members of the flavonoid family. Different flavonoids have different chemical properties but share the ability to function as antioxidants. However, the strength of this antioxidant activity varies widely between the different flavonoid types and depends on other structural features such as glycosylation patterns in the rings.

Table 4.1. Typical flavonoid compounds in major subgroups of selected fruits, vegetables, and grains[a]

Phytochemical groups/subgroups	Representative compounds	Food sources
Benzoic acids	Gallic acid, protocatechuic acid	Apples, small berry fruits, wine, cereal grains[d]
Cinnamic acids	Ferulic acid, chlorogenic acid	Apples, small berry fruits, wine, cereal grains[d]
Chalcones	Xanthohumol	Hops, beer[c]
	Phloretin	Apples[e]
Flavones	Apigenin, luteolin	Bell peppers, brussel sprouts, cabbage, cauliflower, celery, chives, kale, lettuces, spinach, peppers, tomatoes, watercress
	Tangeretin, nobiletin	Citrus fruits
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Apples, asparagus, berries, broccoli, cabbages, chives, cranberries, grapes, kale, onions, peppers, spinach, Swiss chard, tomatoes, watercress, cereal grains
Flavanones	Naringenin, hesperetin	Oranges and other citrus fruits
Flavanonols	Taxifolin	Citrus fruits[b]
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Blueberries, blackberries, cranberries eggplants, pomegranates, plums, raspberries, red onions, red potatoes, red grapes, red radishes, strawberries, other red-purple fruits and vegetables, black rice, blue and purple wheat
Flavanols/procyanidins/ theaflavins	Catechin, epicatechin, and their gallic acid esters	Apples, grapes, plums, pears, mangoes, okra, peaches, Swiss chard, berries, fruits and vegetables in general, green tea, peanut
	Monomers, dimers (procyanidin B1, B2), and oligomers	Apples, cherries, berries, grapes, peaches, pears, cocoa beans
	Theaflavin	Black tea
Isoflavonoids	Diadzein, genistein, formononetin, biochanin A	Soybean and soy products, red clover, alfalfa sprouts, bean sprouts[f]
Stilbenes	Resveratrol	Grapes, red wine
Ellagic acids	Ellagic acid, valoneic acid dilactone	Raspberries, strawberries, walnuts, heartnuts
Lignans	Secoisolariciresinol diglucose, matairesinol	Flax, wheat, barley
Amides	Capsaicin, avenanthramides	Chili peppers, oats
Carotenoids	β-carotene, lycopene, lutein	Peaches, watermelon, tomatoes, spinach, kale, broccoli, oil seeds, ancient grains, pulse crops, carrots, pumpkin, winter squash, potatoes

[a] Information was collected from the USDA flavonoids database release 2.1.
[b] Kawaii et al. 1999.
[c] Zhao et al. 2005.
[d] Robbins, 2003.
[e] Tsao et al. 2003.
[f] Lee et al., 2007.

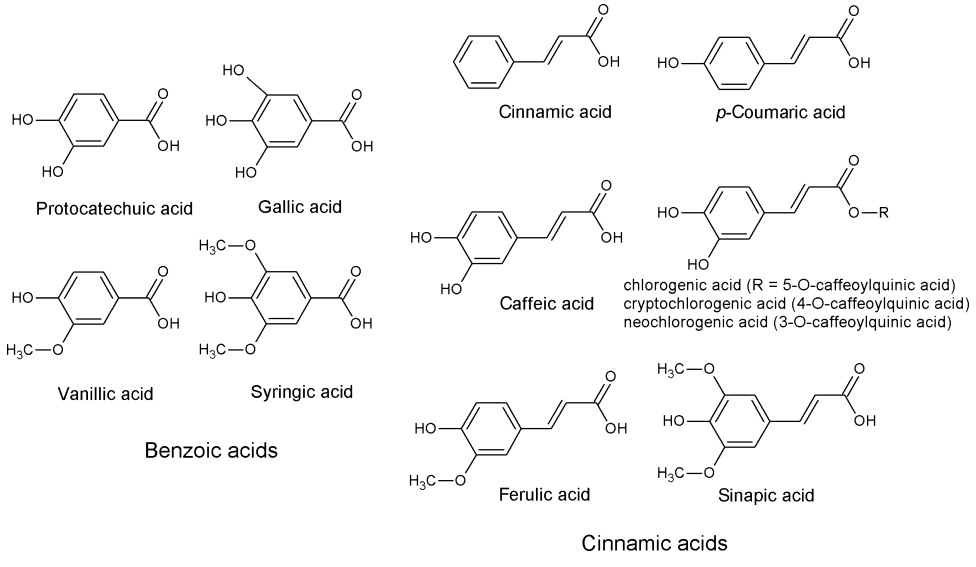

Figure 4.1. Phenolic acids commonly found in food.

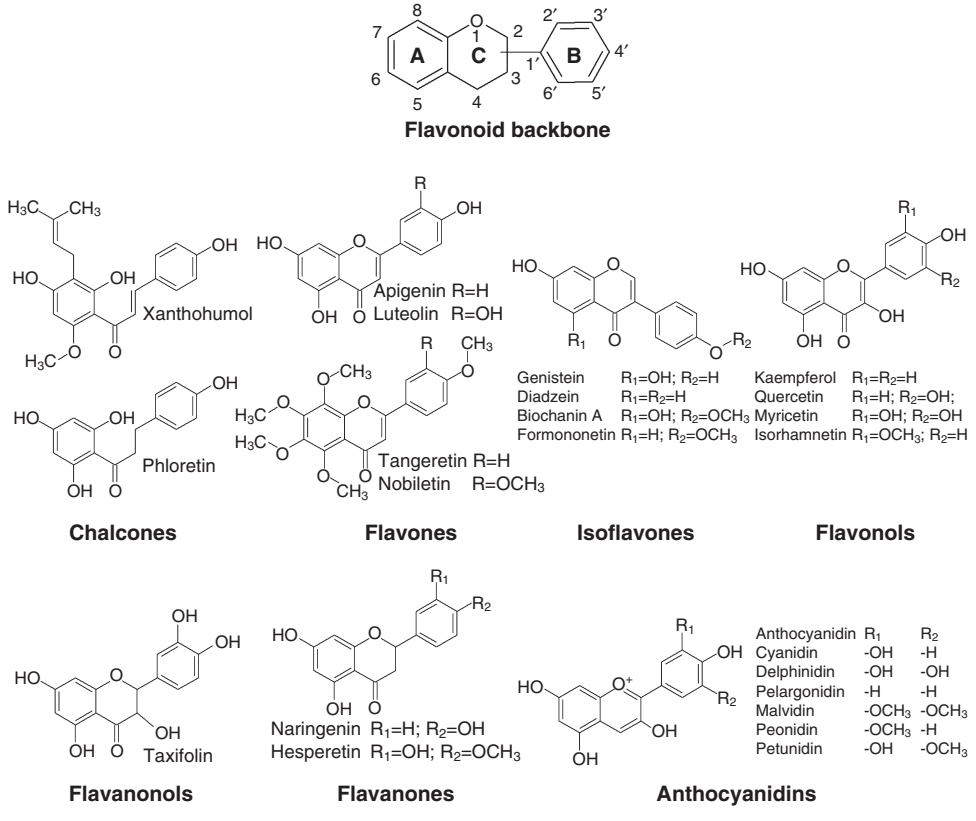

Figure 4.2. Typical flavonoids (excluding catechins).

Flavonoids are aglycones, but these compounds usually exist as glycosides and acylglycosides such as malonylglycosides in fruits and vegetables. C-glycosylflavonoids, where the sugar unit is attached to the flavonoids through carbon–carbon bonding, are also found in plants although less in fruits and vegetables (Harborne, 1994; Anderson and Markham, 2006).

Flavonoids with a B ring attached to position 2 of the C ring can be further categorized into several different subgroups according to structural features of the chromane ring (ring C): flavones, flavonols, flavanones, flavanonols, and anthocyanidins. Among these subgroups, the *O*-glycosides of flavones and flavonols make up one of the largest classes of flavonoid constituents in plants with over 2000 known structures (Williams, 2006). Typical flavonoid subgroups and their distribution in food plants are listed in Table 4.1, and their structures are shown in Figure 4.2.

Chalcones, flavones, flavonols, flavanonols, flavanones, and anthocyanidins

Chalcones and dihydrochalcones and their glycosides have been found in many fruits and vegetables (Tsao et al., 2003; Arabbi et al., 2004); however, occurrence of these open structured flavonoids is generally rare (Tomás-Barberán and Clifford, 2000). Prenylated chalcones such as xanthohumol are found in beers and hops (Zhao et al., 2005) (Fig. 4.2).

Flavones are a large subgroup of flavonoids with apigenin and luteolin as the most widespread flavone aglycones (Valant-Vetschera and Wallenweber, 2006). Flavones have a double bond between positions 2 and 3, and a ketone on position 4 of the C ring (Fig. 4.2). Hydroxyl group(s) can be mostly found at position 5 of ring A, but other positions such as 7 of ring A, 3′ and 4′ of ring B can also occur. Some flavones are polymethoxylated such as tangeretin and nobiletin which are found in the peels of citrus fruits.

Flavonols such as quercetin and kaempferol are nearly ubiquitous in plants. Fruits, vegetables, and grains are the richest dietary sources of these compounds (Valant-Vetschera and Wallenweber, 2006). The only difference between flavonols and flavones is the hydroxyl group on position 3 of ring C of the former. This 3-hydroxyl group is often found to be glycosylated in plants.

Flavanones and flavanonols are considered minor flavonoid subgroups. The structural features of flavanones are the same as flavones except that there is no double bond between positions 2 and 3 in ring C. Flavanonols are basically 3-hydroxyl flavanones. Both of these two subgroups can be structurally highly diverse and multi-substituted (Grayer and Veitch, 2006). Taxifolin is a well-known flavanonol found in citrus fruits (Kawaii et al., 1999).

The red, blue, and purple colors of small berry fruits, red apples, cherries, purple onion, black rice, and blue wheat are from the various anthocyanidins (Fig. 4.2). Anthocyanidin glycosides are often referred to as anthocyanins which are the actual form that exist in plants. Although more than 500 anthocyanins are known, these compounds are based on 31 monomeric anthocyanidins, among which cyanidin, delphinidin, and pelargonidin are the most widely found (Anderson and Jordheim, 2006). Chemically, anthocyanidins are flavylium cations, and their colors change depending on the pH. In acidic conditions, anthocyanidins are red, but as the pH increase to basic, they change to blue. The color of anthocyanidins can also be affected by how the various hydroxyl groups of ring A or B are acylated or methylated. Glycosylation pattern (i.e., type of sugar), acylation with phenolic acids of the sugar unit, and also position can affect the color. Anthocyanin-rich vegetables such as purple cauliflowers and black/purple carrots are gaining popularity due to their enhanced antioxidant activities.

Catechins (Flavan-3-ols), procyanidins, and theaflavins

Catechins are also interchangeable with the term flavanols, or more strictly speaking, flavan-3-ols, because the hydroxyl group is almost always attached to the position 3 carbon of ring C. Catechins have two epimers depending on the stereo configurations of the bond between ring B and the position 2 carbon, and the hydroxyl group on position 3. These two epimers, (–)-epicatechin and (+)-catechin, and their respective derivatives, epigallocatechin and gallocatechin, are together categorized as catechins (Fig. 4.3). Gallocatechin and epigallocatechin contain an extra hydroxyl group on ring B. Catechins are often found in the skins of fruits and certain vegetables. Tea also contains various forms of catechins

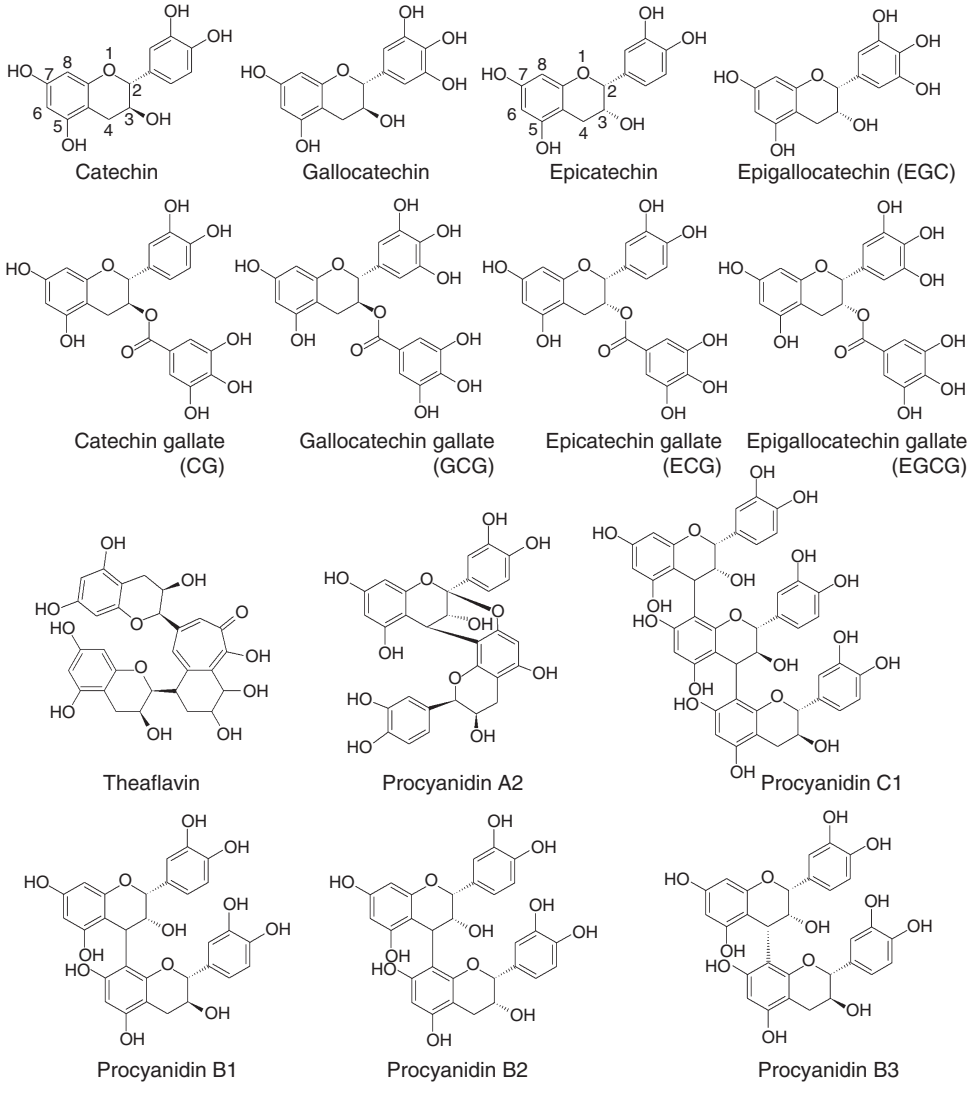

Figure 4.3. Monomeric and typical dimeric and oligomeric catechins.

(Harnly et al., 2006). Green tea in particular contain many gallic acid esters of the catechins, whereas black tea contain theaflavin and its derivatives.

One important characteristic of the flavanols is their ability to form polymers. Polymeric forms of flavanols are often referred to as procyanidins, which are also called proanthocyanidins or condensed tannins as opposed to hydrolyzable tannins (e.g., gallotannins and ellagitannins, esters of glucose, and gallic acid or ellagic acid, respectively). Procyanidins usually contain 2–60 units of monomeric flavanols, and the polymerization most often occurs via a carbon–carbon bond between the position 8 carbon (C8) of the terminal unit and C4 of the extender. The four most common dimers are procyanidins B-type procyanidins (e.g., B1, B2, B3, and B4) with their modes of coupling demonstrated in Figure 4.3. Further polymerization yield the linear 4,8 polymers. Procyanidins with 2–7 catechin units are oligoprocyanidins (OPCs) (e.g., Procyanidin C1). Flavanols also form A type procyanidins in which flavanol monomers are doubly linked (e.g., procyanidin A2) (Prior et al., 2001; Liu et al., 2007). More information on the different types of procyanidins can be found in reviews by Prior et al. (2001), Xie and Dixon (2005) and Ferreira et al. (2006).

Isoflavones

Isoflavones are a unique subgroup of flavonoids which have their B ring borne on position 3 of the C ring (Fig. 4.2). Unlike other flavonoids, isoflavonoids are mainly found in the legumes. Commonly consumed fruits and vegetables are not good sources of isoflavones; however, soy or soy products, and certain specialty vegetables such as soybean and alfalfa sprouts, do contain large amounts of isoflavones, including genistein and daidzein (Lee et al., 2007).

4.4.1.3 Other Polyphenols

In addition to the phenolic acids and flavonoids, several other polyphenolic groups have been known as strong antioxidants. One of the best-known polyphenols in red grape and wine are the stilbenes which are represented by resveratrol (Fig. 4.4). Small berries such as raspberry and strawberry contain high concentrations of ellagic acid and its derivatives. These compounds (ellagitannins) are also found in nuts, particularly in the seed coats of nuts. Ellagic acid and valoneic acid dilactone (Fig. 4.4) have been reported in a Canadian tree nut, the heartnut (Li et al., 2006b). Lignans are another important group of polyphenols that are often found in cereal grains and flax seed, and again mostly in the bran or seed coat in a highly complex form. Free lignans are released only upon acid or base hydrolysis of the lignan complex, or through the digestive tract in animals. Lignans are not only strong antioxidants but also act as precursors of mammalian lignans. Secoisolariciresinol diglucoside (SDG) and matairesinol (Fig. 4.4) are two important lignans for human health.

4.4.2 Amides

Two groups of amides are of significance in terms of being strong antioxidants: capsaicinoids in chili peppers, and avenanthramides in oats (Fig. 4.5). Capsaicinoids such as capsaicin is responsible for the hotness of chili peppers, but they have also been found to be strong antioxidants, anti-inflammatory agents, and oxidative defense system modulators (Surh, 2002). Avenanthramides are antioxidants that can inhibit low-density lipoprotein (LDL) oxidation (Chen et al., 2004).

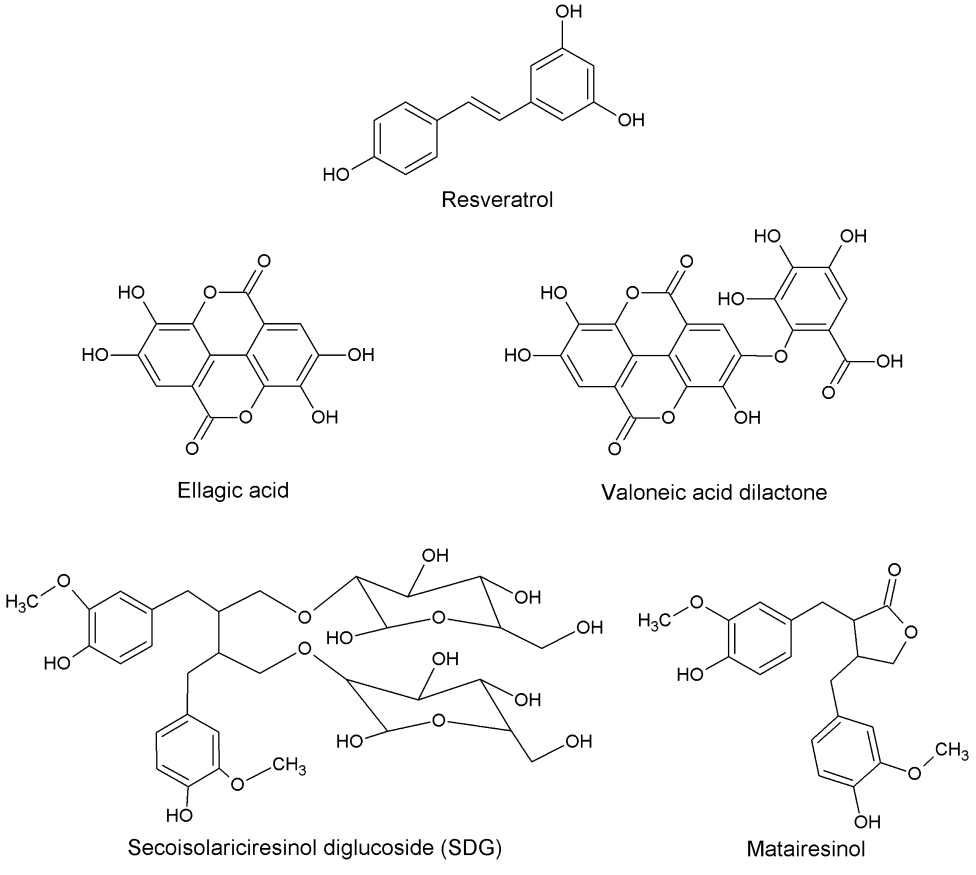

Figure 4.4. Other commonly found polyphenols in food.

4.4.3 Carotenoids

Carotenoids are tetraterpenoids. Like all terpenoids, they are produced in plants through the isoprenoid or terpenoid pathway. The C5-isoprene unit is the building block of all isoprenoids. Monoterpenoids are synthesized from two isoprene units, and the 4-unit geranylgeranyl diphosphate (GGDP) is the precursor to the diterpenoids. Carotenoids are synthesized from two GGDP molecules through a head-to-head condensation catalyzed by the enzyme phytoene synthase. Phytoene is the precursor to lycopene whose highly conjugated double bond system is formed by phytoene desaturase and ζ-carotene desaturase. Lycopene is key to two main groups of carotenoids: (1) β-carotene and its derivative xanthophylls such as zeaxanthin, violaxanthin, and neoxanthin; and (2) one β- and one ε-ring systems such as α-carotene and lutein (Del Villar-Martinez et al., 2005). At least 500 carotenoids have been identified so far, mostly from plants and algae (Delgado-Vargas et al., 2000). Figure 4.6 shows typical carotenoids found in food. While all carotenoids are good antioxidants and scavenge singlet oxygen and other ROS (Stahl and Sies, 1997), their activities are not the same (Simic, 1992). Xanthophylls (oxygen-

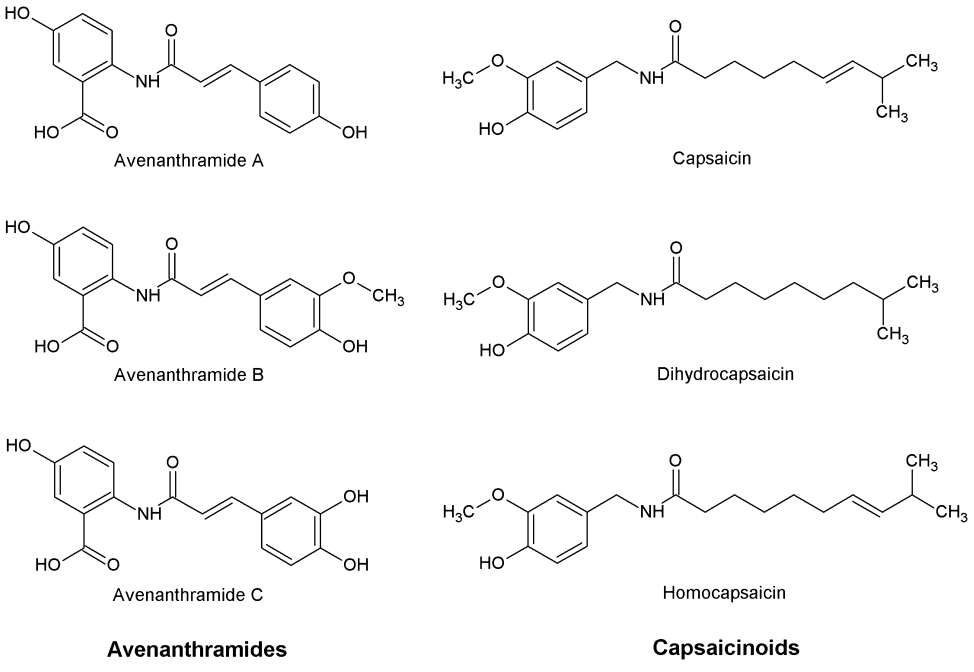

Figure 4.5. Amide antioxidants.

containing carotenoids) are in general considered to be stronger antioxidants. The essential role of carotenoids as major dietary sources of vitamin A has been known for many years.

4.4.4 Mechanism of antioxidant action

A high dietary intake of fruits, vegetables, and whole grains has been linked to reduced risks of many chronic diseases including cancer, cardiovascular disease, and chronic inflammation. Many of these conditions are caused by oxidative stress from reactive oxygen and nitrogen species. Fruits, vegetables, and whole grains are rich sources of phytochemicals that have strong antioxidant activities *in vitro*. While these *in vitro* antioxidant model systems are limited in terms of similarity to the mechanisms of antioxidant actions in a biological system, collectively, they adequately portray how phytochemicals might function as antioxidants.

4.4.4.1 Antioxidant assay models

Antioxidant activities or capacities of phytochemicals are measured *in vitro* using mainly chemical models. While many different models have been used by researchers, most of the model systems are based on either hydrogen atom transfer (HAT) or single electron transfer (ET) mechanisms and are discussed later in this chapter.

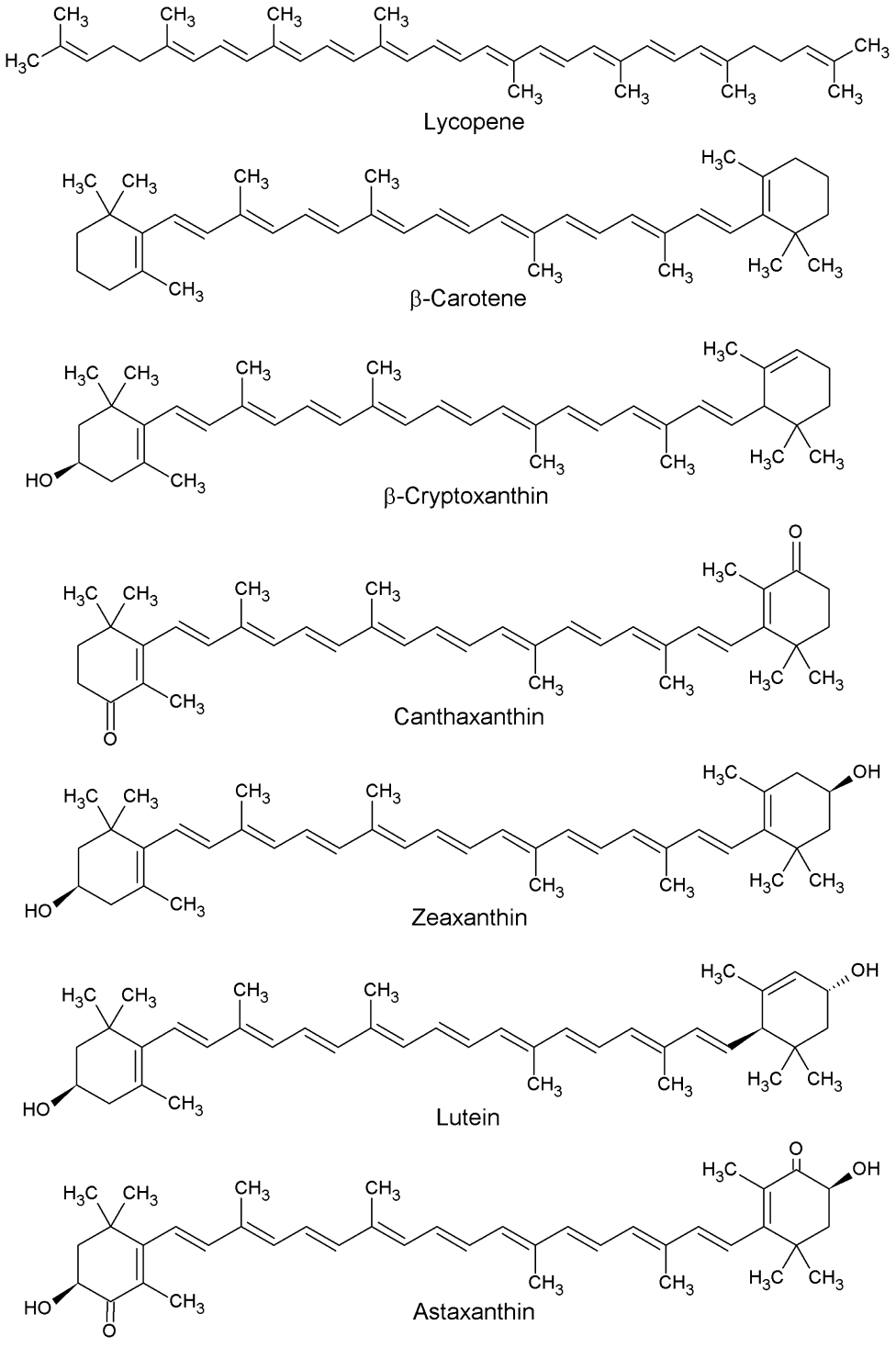

Figure 4.6. Carotenoids found in food.

4.4.4.2 Biologically relevant antioxidant mechanisms

Although radical scavenging activity is one of the most recognized mechanisms of antioxidant actions, other mechanisms are also known. Disilvestro (2001) proposed six different potential mechanisms, including (1) direct radical scavenging; (2) downregulation of radical production; (3) elimination of radical precursors; (4) metal chelation; (5) inhibition of xanthine oxidase; and (6) elevation of endogenous antioxidants.

Direct radical scavenging antioxidants are chain-breaking agents of lipid peroxidation. Chain breakers donate a proton to the free radicals, neutralizing the radicals and themselves becoming stable (less reactive) radicals, thus stopping the chain reactions. Vitamin E, polyphenols, and most recently carotenoids have been found to act as chain breakers of the free radical chain reactions (Rice-Evans et al., 1996; Pietta, 2000; Guo et al., 2009). Indirectly, phytochemical antioxidants act as preventive agents that suppress the generation of free radicals and reduce the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals.

Antioxidant activities by phytochemicals, particularly carotenoids, are known via the quenching of singlet oxygen which causes photosensitized oxidation (Hirayama et al., 1994). Other polyphenols are known as metal chelators. Chelation of transition metals such as Fe^{2+} can directly reduce the rate of Fenton reaction thus preventing oxidation caused by highly reactive hydroxyl radicals. Polyphenols are known to bind to such metal ions (Pietta, 2000; Perron and Brumaghim, 2009).

Phytochemicals such as polyphenols can also exert their antioxidant action indirectly by upregulating antioxidant enzymes such as GPx, CAT, and SOD, and by inhibiting the expression of enzymes such as xanthine oxidase that are involved in the generation of ROS (Du et al., 2007). Curcumin and flavonoids have been shown to upregulate intracellular GSH synthesis (Myhrstad et al., 2002; Biswas et al., 2005) and increase antioxidant enzyme activities (Molina et al., 2003; Li et al., 2006a) (Table 4.2). Recent data demonstrate that the increase in GSH levels in various cell types treated with polyphenols depends on the increased transcription of the γ-GCS gene (Myhrstad et al., 2002; Moskaug et al., 2005) and that dietary polyphenols can stimulate the transcription of antioxidant and detoxification defense systems through ARE elements (Dinkova-Kostova and Talalay, 1999; Chen et al., 2000). It has been suggested that polyphenols influence the pathways that regulate ARE activation by modifying the capability of Keap1 in sequestering Nrf2 and/or activate MAPK proteins (ERK, JNK, and p38) involved in Nrf2 stabilization (Masella et al., 2005). Nrf2 would thus translocate to the nucleus and transactivate the ARE-containing promoter of antioxidant genes (SOD, CAT, γ-GCS, GST, GR, and GPx). Oxidized or reactive forms of the major green tea polyphenol, (–)-epigallocatechin-3-gallate (EGCG), are thought to conjugate with GSH, thereby disrupting the cell's redox status and activating protein kinase pathways which trigger Nrf2 phosphorylation (Andreadi et al., 2006; Wu et al., 2006). Alternately, reactive EGCG may directly interact with Cys residues of Keap1 to stimulate Nrf2 dissociation. ROS produced by EGCG auto-oxidation could also oxidize Cys thiols of Keap1. These events liberate Nrf2 and facilitate the nuclear translocation of Nrf2 (Na and Surh, 2008).

While the overwhelming research activities have been focused on the antioxidant properties of phytochemicals, there is an emerging view that phytochemicals, particularly polyphenols, and their *in vivo* metabolites, do not only act as conventional hydrogen- or electron-donating antioxidants but may also exert modulatory actions in cells through actions at protein kinase and lipid kinase signaling pathways (Williams et al., 2004). The

Table 4.2. Antioxidative stress properties of selected phytochemicals (nonexhaustive list)

Phytochemical	Mechanism of action	References
Resveratrol	Concentration and/or time-dependent induction of SOD, CAT, GSH, GR, GPx, and GST activity. Increased mRNA expression of CAT and GSTA1	Li et al. (2006a)
Curcumin	Increased GSH levels and γ-GCS activity in astrocytes	Biswas et al. (2005)
	Induction of GSH; inhibition of NFκB and interleukin-8 (IL-8)	Lavoie et al. (2009)
Quercetin	Pretreatment with quercetin results in significant increase in hepatic GPx, GR, SOD, and CAT activities, and in GSH concentrations of mice chronically exposed to ethanol	Molina et al. (2003)
EGCG	EGCG pretreatment of skin restored GSH level and GPx activity after UV exposure	Katiyar et al. (2001)
	Induction of GST activity in rat liver treated with hydrogen peroxide by portal vein perfusion	Chou et al. (2000)
	Potent induction of ARE-mediated gene expression	Chen et al. (2000)
	Induced expression of γ-GCS and γ-glutamyltransferase 1 in Nrf-2 wild-type mice	Shen et al. (2005)
Green tea polyphenols	Oral feeding for 30 days induced expression of GPx, CAT, and quinone reductase in the small bowel, liver, and lung of SKH-1 hairless mice	Khan et al. (1992)
Flavonoids	Increase in GSH and mRNA expression of γ-GCS	Myhrstad et al. (2002)

effect on biomarkers involved in these pathways can lead to changes in cellular functions, thus the potential health benefits. Williams et al. (2004) also suggest that "a clear understanding of the mechanisms of action of flavonoids, either as antioxidants or modulators of cell signaling, and the influence of their metabolism on these properties are key to the evaluation of these potent biomolecules as anticancer agents, cardioprotectants, and inhibitors of neurodegeneration." In addition, phytochemicals do not usually act alone and can actually function as co-antioxidants, and are involved in the regeneration of essential vitamins (Zhou et al., 2005). Therefore, phytochemicals are not simply antioxidants, but have a multitude of functions which contribute significantly to disease prevention and amelioration.

4.5 ANTIOXIDANT AMINO ACIDS, PEPTIDES, AND PROTEINS

Amino acids, peptides, and proteins have biological activities beyond that of nutrition. Gastrointestinal digestion or chemical, enzymatic, or bacterial hydrolysis of food proteins result in the production of bioactive amino acids and peptides. Bioactive peptides and whole proteins with antioxidant and antioxidative stress properties have been reported from a range of foods including dairy, egg, soy, potato, and gelatin (Elias et al., 2008). The

antioxidant properties of food molecules are most commonly assessed by the inhibition of lipid peroxidation (a linoleic acid auto-oxidation system is frequently used), the ability to scavenge free radicals, and the reducing power. Although most of these assays are evaluated in food systems, the results provide an idea of what might occur in the human body. *In vitro* and *in vivo* models are employed for the determination of antioxidative stress activities, and sometimes, animal disease models are used. In these studies, researchers examine changes in GSH and GSH-synthesizing enzymes, as well as antioxidative enzyme gene expression and activities.

4.6 MECHANISM OF ACTION OF ANTIOXIDANT AND ANTIOXIDATIVE STRESS AMINO ACIDS, PEPTIDES, AND PROTEINS

4.6.1 Amino acids

The vulnerability of amino acids to ROS oxidation is primarily determined by the R-group side chain, but solvent accessibility and other amino acid residues in the surrounding area influence oxidative susceptibility as well (Davies and Dean, 1997). In theory, all amino acids are potentially oxidizable, but the most susceptible to hydrogen abstraction include those with nucleophilic sulfur-containing side chains such as cysteine and methionine, or aromatic side chains like tryptophan, tyrosine, and phenylalanine (Stadtman, 1993; Elias et al., 2008). The imidazole side chain of histidine is also easily oxidized. Amino acids, peptides, and proteins in the body can be present at high concentrations and can therefore act as antioxidants and contribute to the overall ROS scavenging activity (Droge, 2002). Methionine is particularly important since Met supplementation to mice fed a protein-free diet was able to decrease ROS levels, increase GSH concentrations, and lower protein oxidation, thereby restoring the liver's antioxidant defenses.

Certain amino acids have been recently demonstrated to also exhibit antioxidative stress properties (Table 4.3). Pretreatment of H_2O_2-stimulated human intestinal Caco-2 cells with Cys, Val, Ile, Leu, Trp, His, Lys, and Ala was found to significantly inhibit secretion of the oxidative stress biomarker, IL-8 (Son et al., 2005; Katayama and Mine, 2007). Amino acids with branched chains such as Val, Ile, and Leu enhanced GST and CAT activities, while Trp, His, and Lys increased GST activity. Alanine boosted GR activity, and cysteine enhanced both γ-GCS activity and cellular GSH levels. The bioactivity of cysteine is not surprising since it is the rate-limiting substrate for GSH synthesis. Cystine, histidine, glutamine, and threonine jointly protected mouse astrocytes from Zn-induced oxidative stress (Ralph et al., 2010). In addition, the supplementation of methionine to a protein-free diet (PFD) protected the mouse liver from the increase in ROS and oxidized protein, and the decrease in GSH associated with dietary amino acid deprivation (Ronchi et al., 2010).

4.6.2 Peptides and proteins

The antioxidant capacity of peptides and proteins is also determined by the functional R-group, except in this case the conformation of molecule and the intramolecular bonds affect the degree of oxidation (Davies and Dean, 1997). Oxidation of amino acid residues can result in radicals which can be propagated to adjacent side chains, thereby increasing damage to the rest of the molecule. Antioxidant nutraceuticals in the form of bioactive

Table 4.3. Mechanism of action of amino acids

Amino acids	Mechanism of action	References
Cysteine Methionine	Free radical scavenging, metal chelation, hydrogen donor	Stadtman (1993); Elias et al. (2008)
Tryptophan Tyrosine Phenylalanine Histidine	Histidine, cystine, glutamine, and threonine were found to collectively protect cultured mouse astrocytes from zinc-induced oxidative stress. These amino acids were proposed to act by chelating zinc, serving as GSH precursors, or converting to tricarboxylic acid cycle intermediates.	Ralph et al. (2010)
	Supplementation of methionine to a protein-free diet in mice decreased ROS content, lowered oxidized protein concentrations, and increased GSH levels in the liver.	Ronchi et al. (2010)
Cysteine Valine Isoleucine Leucine Tryptophan Histidine Lysine Alanine	Inhibition of IL-8 in hydrogen-peroxide induced Caco-2 human intestinal carcinoma cells.	Son et al. (2005); Katayama and Mine (2007)
Valine Isoleucine Leucine	Increased GST and CAT activities	Katayama and Mine (2007)
Tryptophan Histidine Lysine	Enhanced GST activities	
Alanine	Increased GR activity	
Cysteine	Enhanced γ-GCS activity and cellular GSH levels	

peptides and proteins are important since they can be preferentially oxidized if they are more oxidatively labile than unsaturated fatty acids and if they are closer to the site of ROS generation (Elias et al., 2008). In this manner, they can protect membrane lipids from oxidation. Proteins, and especially protein hydrolysates, also act by chelating pro-oxidative transition metals and reducing hydroperoxides (Dean et al., 1997; Elias et al., 2008).

4.6.2.1 Peptides

Peptides such as those shown in Table 4.4 are more likely to have bioactive properties compared to the native protein from which the peptides were derived. Soy peptides have increased antioxidant activities compared to intact proteins and in fact, the enzymatic digests of soy β-conglycinin and glycinin had 3–5 times higher radical-scavenging activities compared to the unhydrolyzed proteins (Chen et al., 1998). It is believed that during hydrolysis, the unfolding of the protein structure exposes amino acids previously unavailable within the parent protein. Some soy peptides consist of a Pro-His-His active center and these His-containing peptides likely contributed to the antioxidant activity by chelating metals, quenching active oxygen molecules, and scavenging hydroxyl radicals (Chen et al., 1995, 1996). Antioxidant amino acids such as His, Tyr, Trp, Met, and also Lys increase the

Table 4.4. Examples of antioxidant and antioxidative stress peptides from egg, soy, and milk (nonexhaustive list)

Peptide description	Mechanism of action	References
Egg yolk peptides	Enzymatic hydrolysis of egg yolk proteins scavenged superoxide, hydroxyl, and DPPH radicals, and inhibited TBARS formation.	Sakanaka and Tachibana (2006)
	Delipidated egg yolk peptides prepared using Orientase (EC 3.4.11.12) inhibited linoleic acid oxidation.	Sakanaka et al. (2004)
	Hen egg yolk phosvitin phosphopeptides (PPP3) inhibited H_2O_2-induced IL-8 secretion, suppressed MDA formation, increased GSH levels, and elevated GR, γ-GCS, GST, and CAT activities. γ-GCS-HC (heavy chain) mRNA was also significantly elevated by PPP3.	Katayama et al. (2006, 2007)
	Egg yolk peptides reduced H_2O_2-induced IL-8 secretion in Caco-2 cells, and increased GSH and γ-GCS mRNA expression and activity, CAT and GST activities, and reduced protein and lipid oxidation in the duodenum, jejunum, ileum, and colon in a porcine model of intestinal oxidative stress.	Young et al., (2010)
Soy peptides	Soy protein hydrolysates inhibited thiobarbituric acid reactive substances (TBARS) and inhibited liposome oxidation.	Peña-Ramos and Xiong (2002)
	Antioxidant peptides identified from the proteolytic digestion of soy protein consist of a Pro-His-His active center; His-containing peptides likely contributed to the antioxidant activity by acting as a metal chelator, active oxygen quencher, and hydroxyl radical scavenger.	Chen et al. (1995, 1996, 1998)
	A fermented soybean product, Douchi, enhanced SOD activity in the liver and kidney, increased CAT activity in the liver and GPx activity in the kidney.	Wang et al. (2008)
Milk peptides	Whey protein hydrolysates prepared from protease F digestion prevented TBARS formation.	Peña-Ramos and Xiong (2001)
	Casein hydrolysates scavenged superoxide anions, DPPH radicals, and hydroxyl radicals.	Suetsuna et al. (2000)

oxidant quenching potential of the peptides (Saito et al., 2003). Soy protein hydrolysates were also found to inhibit thiobarbituric acid reactive substances (TBARS) and liposome oxidation (Peña-Ramos and Xiong, 2002). When cholesterol-fed rats were supplemented with a fermented soybean food (Douchi), there was reduced lipid peroxidation and increased antioxidant enzyme activities in the liver and kidney (Wang et al., 2008).

Egg yolk protein (EYP) hydrolysates were shown to scavenge ROS radicals, inhibit TBARS formation, and prevent lipid peroxidation (Sakanaka et al., 2004; Sakanaka and Tachibana, 2006). Egg yolk peptides prepared from the alcalase and protease N digestion of delipidated yolk proteins have both *in vitro* and *in vivo* antioxidative stress properties (Young et al., 2010). EYP significantly reduced IL-8 in H_2O_2-stimulated Caco-2 cells. In piglets given intraperitoneal infusions of H_2O_2, EYP treatment increased GSH and γ-GCS mRNA expression and activity, significantly increased antioxidant enzyme activities, in particular CAT and GST activities, and reduced protein and lipid oxidation in the duodenum, jejunum, ileum, and colon. Furthermore, EYP boosted the systemic antioxidant status in blood by increasing the GSH concentration in red blood cells. A hen yolk phosvitin phosphopeptide fraction (PPP3) reduced H_2O_2-induced IL-8 concentrations and decreased malondialdehyde levels *in vitro* (Katayama et al., 2006). Not only were lipid peroxidation by-products reduced, but there was also an increase in intracellular GSH levels, a significant increase in γ-GCS activity, and the expression of γ-GCS heavy subunit mRNA. In addition, intracellular GR, GST, and CAT activities were elevated by PPP3 treatment (Katayama et al., 2007).

Milk peptides are also effective antioxidants. Whey protein hydrolysates prepared from protease F digestion prevented TBARS formation (Peña-Ramos and Xiong, 2001), while casein peptides were efficient scavengers of superoxide anions, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and hydroxyl radicals (Suetsuna et al., 2000).

4.6.2.2 Proteins

Proteins with antioxidant and antioxidative stress properties have also been found in egg, soy, and milk (Table 4.5). In egg, hen yolk phosvitin and lysozyme are proteins associated with metal binding and microbial defense, respectively. Phosvitin consists of almost 50% phosphoserine residues (Clark, 1985), and this polyanionic nature imparts its chelating properties. Phosvitin binds iron and results in the killing of bacteria (Sattar Khan et al., 2000), the prevention of hydroxyl radical formation during UV-induced oxidative stress (Ishikawa et al., 2005), and the inhibition of Fe^{2+}-catalyzed phospholipid oxidation (Lu and Baker, 1986). The ionization of the phosphate group in phosvitin can be affected by environmental pH, which could result in structural modifications and changes in antioxidant activity (Guérin-Dubiard et al., 2007). In fact, phosvitin inhibits catalytic activity of Fe^{2+} and Cu^{2+} at pH 6.1 but not at pH 7.8 (Lu and Baker, 1987).

Lysozyme is a defensin with antimicrobial properties which contributes to host innate immunity. In addition to this function, lysozyme also binds to ROS-generating advanced glycation end products (AGE) and reduces oxidative stress. Mice that were exposed to acute and chronic oxidative stress and supplemented with lysozyme displayed increased systemic GSH : GSSG levels, and decreased AGE levels in the serum and liver (Liu et al., 2006). In addition, daily lysozyme chloride administrations to rats with induced gastric ulcerations reduced gastric oxidative stress and mucosal hemorrhagic damage (Hung et al., 2007).

Soy proteins given to alcohol-fed rats increased hepatic GSH and CAT levels, lowered hepatic lipid peroxidation, and maintained GR activity at near-normal levels. Similarly, peripheral blood mononuclear cells pretreated with whey protein concentrate increased GSH levels following alcohol-induced oxidative damage (Tseng et al., 2008). Whey protein and casein-fed rats had significantly higher hepatic GSH concentrations during chemically induced intestinal tumor development compared with soybean or red meat-fed rats

Table 4.5. Examples of antioxidant and antioxidative stress proteins in egg, soy, and milk (nonexhaustive list)

Protein source	Mechanism of action	References
Egg	Hen egg yolk phosvitin binds iron and prevents hydroxyl radical formation in the Fenton reaction system and prevents UV-light induced oxidative stress.	Ishikawa et al. (2004, 2005)
	Hen egg lysozyme fed to mice exposed to acute and chronic oxidative injury resulted in the suppression of ROS and a higher systemic GSH : GSSG ratio.	Liu et al. (2005)
Soy	Simultaneous feeding of soy proteins to alcohol-intoxicated rats resulted in increased hepatic CAT activity and GSH content.	Rajasree et al. (2009)
Milk	Whey- and casein-fed rats had higher liver GSH concentrations during dimethylhydrazide tumor induction.	McIntosh et al. (1995)
	A whey-protein diet was more effective than a casein diet in increasing hepatic GSH even after CCl_4-induced oxidative stress.	Balbis et al. (2009)
	Peripheral blood mononuclear cells pretreated with whey protein concentrates and stimulated with alcohol had increased GSH levels.	Tseng et al. (2008)

(McIntosh et al., 1995). A diet of whey protein compared to casein protein also significantly increased hepatic GSH after CCl_4 intoxication. This effect on GSH synthesis is likely due to the abundance of cysteine in whey proteins (Balbis et al., 2009).

4.7 PRODUCTION OF ANTIOXIDANT PEPTIDES

Many food proteins are cleaved into bioactive peptides during pepsin, trypsin, and chymotrypsin digestion (Shahidi and Zhong, 2008). Protein hydrolysis is limited by the substrate specificities of the enzymes and can be influenced by the pH of the food mixture. Peptides can also undergo further cleavage by luminal and intracellular peptidases which may result in a decrease in bioactivity. Despite these possibilities, *in vitro* simulated gastric and intestinal digests of food protein demonstrate that peptides with antioxidant properties can be released (Hartmann and Meisel, 2007). Protein hydrolysates can have different antioxidant activities depending on the peptide size, amino acid sequence, and presence of oxidatively sensitive amino acids. This also holds true for peptides produced from nongastrointestinal means.

Antioxidant peptides can be released from the native protein by the use of heat, acid, or base hydrolysis, or by using proteolytic microorganisms or their proteolytic enzymes. The different substrate specificities of microbial enzymes suggest unique peptides may be formed. The use of proteolytic enzymes in the digestion of a protein results in a wide array of peptides with varying lengths and sequences, and fractionation and purification techniques are often employed for peptide separation (Mine, 2007).

The use of enzyme, temperature, and sample preparation conditions for protein hydrolysis can influence the types of peptides and their subsequent antioxidant potential. Soy protein hydrolysates prepared from native and heat-treated soy protein isolates using a variety of enzymes had varying degrees of hydrolysis (1.7–20.6%) and antioxidant activity (28–65%) (Peña-Ramos and Xiong, 2002). For some foods like soy, it is important to also realize that protein and protein isolates may also harbor bioactive components like isoflavones, saponins, phytic acid, and dietary fibers, which contribute to the antioxidant activities. Unless these bioactive components are removed, it is difficult to confidently pinpoint the antioxidant activities to the peptides.

4.8 RECENT ADVANCES IN ANALYTICAL TECHNIQUES FOR MEASURING ANTIOXIDANT CAPACITY AND OXIDATIVE DAMAGE

Food components with antioxidant and antioxidative stress potentials are typically tested *in vitro* prior to use in animal models of disease. There are numerous interactions that can occur between antioxidants and ROS, including physical and genetic interactions. Chemical antioxidant capacity assays typically measure the potential of the antioxidant to prevent or to halt an oxidative process from proceeding further, while gene expression and activity assays provide information on enzyme regulation and performance. Biologically relevant markers of protein, lipid, and DNA damage can also be measured. These biomarkers demonstrate the antioxidant's efficacy in preventing disease or disease development. In this section, we evaluate recent analytical techniques that are relevant for the evaluation of antioxidant nutraceuticals.

4.8.1 Chemical antioxidant capacity assay

Antioxidant capacity assays broadly assess the ability of an antioxidant to transfer a hydrogen atom (i.e., HAT assays) or to donate an electron (i.e., ET assays) to ROS. These assays measure the ability of the antioxidant to scavenge ROS. They do not measure the entire "capacity" or "power" or "potential" of the antioxidant; therefore, these terms are misleading. However, these assays continue to be referred to in this manner.

4.8.1.1 Major HAT-based assays

HAT assays are based on competitive reaction kinetics. A free radical generator, a probe, and an antioxidant are generally added to a substrate mixture. The antioxidant competes with the probe for oxygen radicals and prevents or delays probe oxidation. The antioxidant capacity of the compound relies on the kinetics of probe inhibition. Different kinetic quantifications are taken depending on the type of HAT assay. Since the probe donates H atoms and is therefore sensitive to oxidation, HAT-based assessments of antioxidants are reflective of the protection which can be expected in biological systems, and these types of assays have been successfully applied to human plasma samples and tissue homogenates (Alho et al., 1998; Kim et al., 2006).

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was first developed by Cao et al. (1993), and the fluorescent protein, B-phycoerythrin (B-PE), was used as a probe. B-PE had several limitations and was soon replaced with fluorescein (Ou et al., 2001). Antioxidant-containing samples compete with the probe for 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-generated peroxyl radicals. Fluorescence decay curves are constructed and the area under the kinetic curve (AUC) is used for the calculation of the ORAC value which is expressed in Trolox equivalents.

The ORAC assay can measure both hydrophilic and lipophilic antioxidant capacity by the addition of randomly methylated *alpha*-cyclodextrin (RMCD) (Huang et al., 2002). An exclusively lipophilic ORAC assay was also developed using the lipid-soluble probe, 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 665/676), and the peroxyl radical generator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVM), and can be carried out in organic media or liposomes (Naguib, 1998). Although the ORAC assay utilizes peroxyl radicals, other radicals such as H_2O_2 and peroxynitrite can be substituted (Chung et al., 2001; Ou et al., 2002).

Total peroxyl radical-trapping antioxidant parameter (TRAP) assay

Wayner et al. (1985) first developed the TRAP assay to determine the antioxidant status in human plasma. This assay has since been used extensively in the measurement of antioxidant capacity in human plasma or serum (Alho et al., 1998; Leinonen et al., 1998) and tissue homogenates (Kim et al., 2006). The TRAP assay also monitors the ability of the antioxidant to interfere with peroxyl radical, but in this case, the lag time in the reaction kinetics is important. R-phycoerythrin (R-PE) is used as a fluorescent probe, Trolox as an internal standard, and TRAP values are expressed as Trolox equivalents (Huang et al., 2005). Another probe, dichlorofluorescein-diacetate (DCFH-DA), has also been applied to the TRAP assay (Valkonen and Kuusi, 1997).

Crocin bleaching assay

The crocin bleaching assay utilizes crocin as a probe and has been used to measure antioxidant capacity in phenolic compounds and in plasma (Bortolomeazzi et al., 2007; Uberos et al., 2007). Crocin is a saffron-derived carotenoid that is bleached in the presence of AAPH-generated peroxyl radicals (Bors et al., 1984). Antioxidants compete with crocin for peroxyl radicals, and crocin bleaching values are expressed as a ratio of initial crocin bleaching rates in the absence and in the presence of an antioxidant-containing test sample. This assay was modified to a 96-well microplate format and instead of using kinetic values, a fixed time was defined, and values were expressed as inhibition percentage (Lussignoli et al., 1999).

Total oxidant scavenging capacity (TOSC)

In the TOSC assay, peroxyl radicals thermally generated by AAPH oxidize α-keto-γ-methiolbutyric acid (KMBA) to ethylene. This reaction takes place in a closed chamber, and ethylene formed in the headspace is analyzed by gas chromatography (GC) (Winston et al., 1998). Other oxidants like the hydroxyl radical and peroxynitrite can be introduced into the TOSC assay by incorporating an iron plus ascorbate-driven Fenton reaction and by decomposition of 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), respectively

(Lichtenthaler and Marx, 2005). Similar to the ORAC assay, the AUC is used to quantify the inhibition of ethylene formation as a function of time. TOSC values are expressed as relative AUC and have recently been used for the analysis of fish liver homogenates (Chesman et al., 2007) and strawberries (Shin et al., 2007).

4.8.1.2 ET-based assays

The main assumption in ET-based assays is that the ability of an antioxidant to donate an electron—its reducing capacity—is on par with its antioxidant capacity (Benzie and Strain, 1999). These assays are simplistic since the oxidant is a probe that changes color upon reduction by an antioxidant. The amount of color change is directly correlated with the antioxidant concentration and the end point is taken at the termination of the color change. The antioxidant capacity is calculated from the slope of the line in an absorbance change versus antioxidant concentration plot. ET assays usually include a reference antioxidant, and antioxidant capacity values are accordingly expressed as Trolox equivalence and gallic acid equivalence.

2,2′-Azinobis(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) assay

The ABTS assay is also known as the Trolox equivalent antioxidant capacity (TEAC) assay since values are compared to that of Trolox. In recent TEAC assays, the $ABTS^{\bullet-}$ radical is generated by the persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$). The $ABTS^{\bullet-}$ radical is dark green, but once it accepts an electron from the antioxidant, becomes colorless and can be measured at 600–730 nm. The ABTS or TEAC value is expressed as the concentration of antioxidants which results in the same percent change of absorbance of the $ABTS^{\bullet-}$ radical as that of 1 mM Trolox.

This assay is widely popular for the screening of food-derived antioxidants. Since the $ABTS^{\bullet-}$ radical is miscible in water and organic solvents, the antioxidant capacity of both hydrophilic and lipophilic samples can be assessed (Magalhães et al., 2008).

DPPH assay

The DPPH assay is based on the reduction of the 2,2-diphenyl-1-picrylhydrazyl nitrogen radical ($DPPH^{\bullet}$) by antioxidants (Blois, 1958). The stable DPPH radical is dark purple, but upon reduction, forms a pale yellow hydrazine which can be monitored at 515–520 nm. DPPH values can be expressed in terms of EC_{50} (which reflects the amount of sample needed to decrease the initial absorbance of DPPH by 50%), Trolox equivalents, or ascorbic acid equivalents (Magalhães et al., 2008).

Ferric ion reducing antioxidant power (FRAP) assay

In the FRAP assay, antioxidants reduce the ferric-2,4,6-tripyridyl-s-triazine [Fe(III)-$(TPTZ)_2]^{3+}$ complex to the blue ferrous complex [Fe(II)-$(TPTZ)_2]^{2+}$ in an acidic aqueous environment (Benzie and Strain, 1996). The ferrous complex can be monitored at 593 nm, and the absorbance increase can be compared to that of a ferrous ion standard or to a Trolox or ascorbic acid standard. This assay has been widely used to investigate the antioxidant capacity of vegetables, fruits, and cereals, and the results are relatively comparable to that obtained using the DPPH or TEAC assays (Katalinic et al., 2006).

4.8.2 Cell-based antioxidant assays

In vitro antioxidant capacity data do not provide evidence that antioxidants will perform similar functions *in vivo*. In fact, it cannot provide any information on bioavailability, stability, or reactivity *in situ* (Huang et al. 2005). *In vitro* data is useful in the screening of antioxidants; however, if the antioxidants are intended to be used for disease prevention, they must be tested in at least a cell-based system, if not in an animal model. Various biomarkers of cellular oxidative damage can be used to assess the efficacy of the antioxidant. Cells can be harvested from fresh tissue, but hybridoma cell lines are most often utilized because of convenience and reproducibility.

Cell-based assays can also be used to assess the antioxidative stress properties of antioxidant compounds. Antioxidant enzymes such as CAT, SOD, GSH-related enzymes and even GSH itself or the GSH : GSSG ratio can be measured in cells or in animal models. Cell lines are often used for studying the antioxidant's mechanism of action, and cellular assays using intestinal epithelial cells provide information on the transport of antioxidants.

4.8.2.1 In vivo *markers of oxidative damage*

Oxidative damage of lipid, protein, and DNA can be examined in cell or tissue homogenates, plasma, serum, or urine. While most of these assays are applicable to animal and human samples, cell supernatant and homogenates can also be tested.

Malondialdehyde (MDA)
Malondialdehyde is a popular biomarker of lipid oxidation which predominantly arises from the peroxidation of PUFAs that have two or more double bonds. In a model MDA assay system, a lipid substrate such as ethyl arachidonate or cod liver oil is oxidized by Fenton's reagent. Antioxidant samples and standards inhibit oxidation, decrease the formation of MDA, and result in lower MDA concentrations. The MDA formed in the reaction is derivatized with *N*-methylhydrazine to 1-methylpyrazole (1-MP), which can be monitored by GC (Lee et al., 2003). In biological samples such as plasma, MDA can be derivatized with a chromogenic compound such as *N*-methyl-2-phenylindole to yield a stable chromophore (Castillo et al., 2006).

F2-Isoprostanes
F2-isoprostanes are also important products of lipid peroxidation, and it is believed to be the best biomarker of lipid peroxidation (Halliwell and Gutteridge, 2007e). These compounds are formed from PUFAs with a minimum of three double bonds such as linolenic acid and arachidonic acid. F2-isoprostanes can be detected in human urine and plasma as demonstrated by Morrow et al. (1990). In diseased individuals with atherosclerotic lesions, F2-isoprostanes are elevated in bodily fluids and can be detected by GC–mass spectrometry (GC-MS) and liquid chromatography–tandem MS (Lawson et al., 1999; Li et al., 1999).

Protein carbonyl (PC)
The PC assay is based on the method of Levine et al. (1994) in which the PC content is quantified via the reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form a Schiff base. The corresponding hydrazone can then be analyzed spectrophotometrically between 360–385 nm. This is a convenient colorimetric assay which can be used to measure

oxidized protein in plasma and tissue and cell homogenates. It has been recently used to assess protein damage in the intestinal tissues of pigs subjected to gastrointestinal oxidative stress (Young et al., 2010).

8-Hydroxy-2-Deoxy guanosine (8OHdG)
Oxidative damage of DNA can result in strand breakage, abasic sites, deoxyribose damage, and modification of purine and pyrimidine bases (Halliwell and Gutteridge, 2007e). The most biologically relevant and sensitive biomarker of DNA damage is the modified nucleoside, 8OHdG. The 8OHdG assay has been used to assess the *in vivo* efficacy of phytochemicals, and levels can be detected in the urine, plasma, saliva, or in cell culture and tissue extracts. This modified base can be detected by high performance liquid chromatography–mass spectrometry (HPLC-MS) or by enzyme linked immunosorbent assays (ELISA).

Myeloperoxidase (MPO)
Myeloperoxidase is a peroxidase enzyme which, during inflammation, generates oxidants such as HOCl, H_2O_2, and ·OH as an innate defense mechanism against infection (Proctor, 1996). MPO is present in neutrophil granulocytes and monocytes but rarely in tissue macrophages except in human atherosclerotic lesions (Halliwell and Gutteridge, 2007c). High blood levels of MPO have been associated with an increased risk of cardiovascular disease, and MPO has become a diagnostic tool for heart disease. MPO activity is used to evaluate the effect of antioxidant compounds during the *in vivo* generation of oxidative stress (Kalayarasan et al., 2009; Sriram et al., 2009). Tissue and cell culture MPO activity is determined according to the method of Wei and Frenkel (1993) and utilizes 4-aminoantipyrine/phenol solution as the substrate for the MPO-mediated oxidation by H_2O_2.

4.8.2.2 Determination of GSH and antioxidant enzyme activity and expression

Total GSH can be determined by the Allen et al. (2000) method in which both GSH and GSSG are quantified. In this assay, GSSG is reduced to GSH in the presence of NADPH and GR. The SH group of GSH reacts with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, also known as Ellman's reagent) and forms a yellow colored 5-thio-2-nitrobenzoic acid (TNB) compound which is measured at 405 nm. To examine the GSH : GSSG ratio, GSSG alone is determined using the same assay but GSH is first derivatized with 2-vinylpyridine (Griffith, 1980). The γ-GCS activity can be assayed by the method of Seelig and Meister (1985) by following the oxidation of NADH at 340 nm and 25°C. This assay closely follows the biological formation of γ-glutamylcysteine from glutamate, cysteine, and ATP—which is the first step in GSH synthesis. When ATP is dephosphorylated to adenosine diphospahte (ADP), the ADP is reconverted to ATP by means of pyruvate kinase and phosphoenolpyruvate. The pyruvate that is produced reacts with lactate dehydrogenase and NADH, and in the process oxidizes NADH to NAD^+. γ-GCS activity is expressed in terms of the amount of NADH oxidized/min/mg protein. The γ-GCS mRNA expression can also be determined using a primer for the heavy chain γ-GCS sequence and real-time polymerase chain reaction (RT-PCR).

Antioxidant enzymes serve to neutralize or lessen the effects of ROS; therefore, enzyme activity and gene expression are often determined. This is especially important for antioxidative stress nutraceuticals which can interfere with cell signaling mechanisms and result in the upregulation of GSH synthesis and antioxidant enzymes. Enzyme activities are

preferentially measured instead of protein levels or gene expression since this better reflects the state of the enzymes during oxidative stress. The concentration of enzymes can be misleading since some enzymes could be inactivated by oxidants, while increased mRNA expression does not always equate to more enzymes (Halliwell and Gutteridge, 2007c).

The determination of antioxidant enzyme activities for SOD, CAT, GPx, GR, and GST will be discussed in brief detail since full assay procedures can be found in the references cited. The SOD assay is based on the reaction of water soluble tetrazolium-1 with superoxide anion which forms a colorimetric formazan dye. Since xanthine oxidase reduces O_2 to $O_2^{\bullet-}$ but is converted to H_2O_2 by SOD, the inhibition activity of SOD can be determined by following the decrease in color development. CAT activity is evaluated according to the procedure of Johansson and Borg (1988) by following the decomposition of H_2O_2. This method is based on the reaction of CAT with methanol in the presence of H_2O_2. The formaldehyde produced is measured using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) chromogen. Purpald forms a bicyclic heterocycle with aldehydes, and changes from colorless to a purple color when oxidized. The GPx activity assay monitors the oxidation of NADPH to $NADP^+$ (Wendel, 1981). This is an indirect assay whereby GPx is coupled with GR. GPx reduces H_2O_2 to water by oxidizing GSH to GSSG. GSSG is reduced back to GSH through the oxidation of NADPH. The GR activity assay is a direct assay which follows the decomposition of β-NADPH at 340 nm (Carlberg and Mannervik, 1985). GST activity is determined according to the procedure of Habig et al. (1974) by following the formation of the 1-chloro-2,4-dinitrobenzine (CDNB)-GSH conjugate. These enzyme activity assays can be used for a variety of biological samples including plasma, serum, red blood cell lysates, and tissue and cell homogenates.

4.9 HEALTH BENEFITS OF NUTRACEUTICAL ANTIOXIDANTS

4.9.1 Evidence of antioxidant efficacy in disease states

In both animal and human trials, nutraceutical antioxidants have been found to be effective in the prevention or amelioration of chronic intestinal diseases. A study examining the association between regular multivitamin use (four or more times per week) and colorectal cancer incidence found regular multivitamin users 10 years before study enrollment were at similarly reduced risk whether they were still multivitamin users at enrollment or had stopped (Jacobs et al., 2003). The green tea polyphenol, EGCG has shown strong protective effects against experimentally induced forestomach (Katiyar et al., 1993, 2001) and colon (Han and Xu, 1990) cancers. Inhibitory effects were also observed for EGCG and green tea extracts in duodenal, stomach, and colon carcinogenesis in rodents (Yamane et al., 1996). Furthermore, in animal colitis models, green tea polyphenols alleviated intestinal inflammation and improved GSH levels in the blood (Mazzon et al., 2005; Oz et al., 2005).

Supplementation with cysteine, the rate-limiting substrate for the synthesis of GSH, was demonstrated to attenuate inflammatory responses in a porcine model of colitis (Kim et al., 2009). Hen egg lysozyme treatment in a similar model demonstrated anti-inflammatory and immunomodulatory functions in the restoration of gut homeostasis (Lee et al., 2009). Egg yolk peptides derived from Alcalase and protease N digestion were also found to reduce oxidative stress and promote antioxidative stress mechanisms in an animal model of intestinal oxidative stress (Young et al., 2010).

4.9.2 Failure of antioxidants to demonstrate efficacy

Among the numerous studies purporting the efficacy of dietary antioxidants, there have been some conflicting reports which suggest their ineffectiveness especially in clinical trials. Randomized controlled trials have shown that some antioxidants (β-carotene, selenium, vitamin E, vitamin C) are ineffective in patients with inflammatory bowel disease (IBD) (Geerling et al., 2000; Trebble et al., 2004, 2005; Seidner et al., 2005). A recent meta-analysis could find no evidence of gastrointestinal cancer prevention by antioxidants, with the possible exception of selenium (Bjelakovic et al., 2004). Even in animal models of intestinal carcinogenesis, the efficacy of quercetin (Pereira et al., 1996), black/green tea extracts, and green tea polyphenols (Weisburger et al., 1998a,b) have been questioned. However, these are some exceptions among a large volume of data supporting the beneficial effects of phytochemicals as antioxidants. It should be borne in mind that chronic degenerative diseases are quite complex and attempting to control these with a single component is inherently futile. If the disease mechanism was primarily due to an imbalance in antioxidant system, supplementing vitamins with antioxidant activity should negate that imbalance. The uptake level of several phytochemicals is quite low, and the aggregate plasma concentration of a variety of these ingredients may have a significant contribution to health. More importantly, the phytochemicals can also function as modulators of second messenger systems or receptor function at the concentrations normally attainable after dietary intake. Thus, for nutraceutical antioxidants being ingested, it is important to understand their biological uptake and metabolism in the gastrointestinal tract. A thorough discussion of uptake and bioavailability of nutraceuticals and their mechanisms of action are discussed in later chapters.

A delicate balance exists between oxidant and reducing forces. Trace elements with antioxidant properties such as copper and selenium may become strongly pro-oxidant both *in vivo* and *in vitro* as a consequence of their physical properties (Abuja, 1998; Terada et al., 1999). Vitamins A, C, and E can also become pro-oxidant when present in low concentrations and in the vicinity of transition metal ions (Neuzil et al., 1995). Polyphenols such as resveratrol were also found to catalyze cellular DNA degradation in the presence of transition metal ions like copper (Heiss et al., 2007).

Another key factor which contributes to the failure of some antioxidants in human trials is the timing of antioxidant administration. Antioxidant nutrients may not be able to cure an installed disease, such as a gastrointestinal cancer, but may be able to play a role in preventing the promotion of disease. The detoxification of oxidants is pertinent in the initial reversible phase of tissue injury but after some time, if oxidative stress is prolonged and of a high degree, there is a point beyond which disease development cannot be prevented (Schiller et al., 1993). Therefore, antioxidant supplementation may seem to have little to no effect in some clinical studies where diseases have progressed beyond the "therapeutic window" of treatment.

4.10 CONCLUSION

Oxidative stress plays a large role in the development and propagation of disease. Food components are now being appreciated for their antioxidant and antioxidative stress properties. The discovery of novel nutraceutical antioxidants as well as the understanding of

their mechanism of action offers new ways in which to tackle oxidative stress and avert the development of disease.

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5 Composition and Chemistry of Functional Foods and Nutraceuticals: Influence on Bioaccessibility and Bioavailability

Jissy K. Jacob and Gopinadhan Paliyath

5.1 INTRODUCTION

The health benefits and disease-preventive effects of functional foods such as fruits and vegetables have been related to the presence of several nutraceutical components such as carotenoids, polyphenols (flavonoids, anthocyanins, etc.), fatty acids, terpenes, sulfur components, to name a few (Andersen and Jordheim, 2006; Jackson and Paliyath, Chapter 2). Consumption of functional foods is associated with a reduced risk for cancer and coronary heart disease. Flavonoids and anthocyanins have been reported to exhibit anti-inflammatory, antiallergic, and vasodilatory actions. In addition, they inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and the activity of enzymes such as cyclooxygenase and lipoxygenase, the enhanced activities of which are integral to the inflammation process. Polyphenols such as anthocyanins (Fig. 5.1) exert these effects as antioxidants, free radical scavengers, and chelators of metal ions. Nutraceutical components belonging to several classes exert their effects at the biochemical and molecular levels and may prevent the development of chronic degenerative diseases. Food Guides in several countries recommend personal consumption levels of five to ten servings of vegetables and fruits every day as a part of a healthy diet.

The biological activity of polyphenols *in vivo* is dependent on the absorption, metabolism, and distribution of these compounds and their metabolites within the body after ingestion (Clifford and Brown, 2006; Holst and Williamson, 2008). Age, genetic predisposition, dietary patterns, and so on, may influence the bioefficacy of absorbed nutraceuticals. Despite the presence of high amounts of polyphenols in fruits, their levels of absorption are relatively low. The level of anthocyanins in foods can range from 100 to 5000 mg/kg (Manach et al., 2004). This variability in turn may influence the dietary intake levels of phenolic components that have been estimated to range from nearly 1 g/day (Kuhnau, 1976) to an estimated 3 g/day (Saura-Calixto and Diaz-Rubio, 2007). However, not all of the phenolic components consumed are bioavailable, since this is influenced by the bioaccessibility and the extent of bioavailability of various components (Manach et al., 2004;

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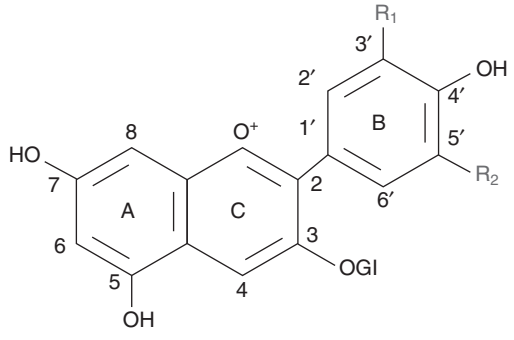

R_1	R_2	Anthocyanin	Color
H	H	Pelargonidin-3-glucoside	Orange
OH	H	Cyanidin-3-glucoside	Orange red
OH	OH	Dephinidn-3-glucoside	Bluish red
OCH_3	H	Peonidin-3-glucoside	Orange red
OCH_3	OH	Petunidin-3-glucoside	Bluish red
OCH_3	OCH_3	Malvidin-3-glucoside	Bluish red

Figure 5.1. Structure and diversity of common anthocyanins. In addition to the simple glucosides, anthocyanins can occur in conjugated forms with acetate and phenolic acids such as caffeic acid, ferulic acid, coumaric acid etc.

Scholz and Williamson, 2007). The bioaccessibility and bioavailability are features that result from the molecular interactions of phenolic components with food matrices such as carbohydrates, proteins, and lipids, and the molecular characteristics that affect the absorption of the molecules in the gastrointestinal tract (GIT). Subsequent to their absorption, the phenolic components may be retained in different parts of the body, or metabolized and excreted, and these factors determine the level of the components available to perform a given function within the body. The plasma and tissue levels of phenolic components are in general in the low micromolar range that may exert an antioxidant effect *in vivo* (Ramirez-Tortosa et al., 2001). Thus, enhancing the bioaccessibility and bioavailability of polyphenols may increase their biological activity, leading to health benefits.

The uptake of polyphenols from fruits and its processed products is highly influenced not only by the composition but also by their availability for absorption. It is generally assumed that polyphenol components such as anthocyanins occur freely in fruits and their processed products, and that their absorption into the body is limited only by their differing affinities toward the transport proteins. However, it is known that a considerable proportion of the polyphenols are not absorbed in the jejunum but pass through into the large intestine where they may be subjected to catabolic breakdown by bacteria and the products are absorbed. It is likely that polyphenols and their breakdown products can be beneficial to the colon due to their antioxidative and anticancer properties. This is evident from several studies (Agullo et al., 1996; Kuntz et al., 1999) that show inverse association between

consumption of fruits and fruit products and lowered incidence of chronic diseases such as inflammatory bowel disease and colorectal cancer. Polyphenols may be carried over to the large intestine by association with nondigestible food matrix. Several types of fruit juices are commonly consumed sources of polyphenols with several health benefits. This chapter summarizes several studies that were conducted to understand the nature of polyphenol–food matrix interactions that may influence the bioavailability and nutraceutical properties of polyphenols.

5.2 POLYPHENOLS AS ANTIOXIDANTS

5.2.1 Free radicals and endogenous antioxidant defense mechanisms

Formation of highly reactive free radicals in the body is a normal consequence of a variety of essential biochemical reactions. A free radical is any atom or molecule that contains one or more unpaired electrons. They are capable of causing damage to cells and tissues. A major internal threat to the cellular homeostasis of aerobic organisms arises from reactive oxygen species (ROS) and the by-products generated from oxygen metabolism. Prime targets for free radical reactions are (1) the unsaturated bonds in membrane lipids which undergo consequent peroxidation resulting in the loss of membrane fluidity, receptor alignment, and potential cellular lysis; (2) enzymes and proteins having sulfur amino acids which become inactivated, cross-linked, and denatured; (3) damage to DNA which subsequently cause mutations that may be carcinogenic; and (4) oxidative damage to carbohydrates altering any of the cellular receptor functions including those associated with hormonal and neurotransmitter responses (Halliwell and Gutteridge, 1985).

Free radicals such as peroxy radicals, superoxide anion, and hydroxyl radicals are responsible for many of these damaging reactions. An antioxidant can be defined as any substance, which when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1985). When free radicals are generated *in vivo*, a vast network of intracellular and extracellular antioxidants act and defend the organism from oxidative damage (Fig. 5.2a,b). Catalase converts H_2O_2 to O_2 and H_2O while superoxide dismutase (SOD) converts the superoxide radical to H_2O_2 and O_2. SOD is found in the chloroplast, mitochondria, and cytosol, whereas catalases are present in peroxisomes. The antioxidant enzymes, SOD, glutathione peroxidase (GPx), and catalase, work within the cells to remove most superoxides and peroxides before they react with metal ions to form more reactive free radicals. GPx is the major enzyme which removes the superoxide generated by SOD in the mitochondria and cytosol by oxidizing glutathione (GSH) into its oxidized form (glutathione disulfide [GSSG]). Peroxidative chain reactions initiated by free radicals that escaped the antioxidant defenses are terminated by chain-breaking water or lipid-soluble antioxidants (Mates et al., 1999).

5.2.2 Diet and exogenous antioxidants (flavonoids)

Diet plays a major role in the body's antioxidant defense mechanism by providing essential nutrients such as vitamin C, E, β-carotene, flavonoids, and essential minerals that act in conjunction with the antioxidant enzymes. For example, SOD contains zinc, and GPx

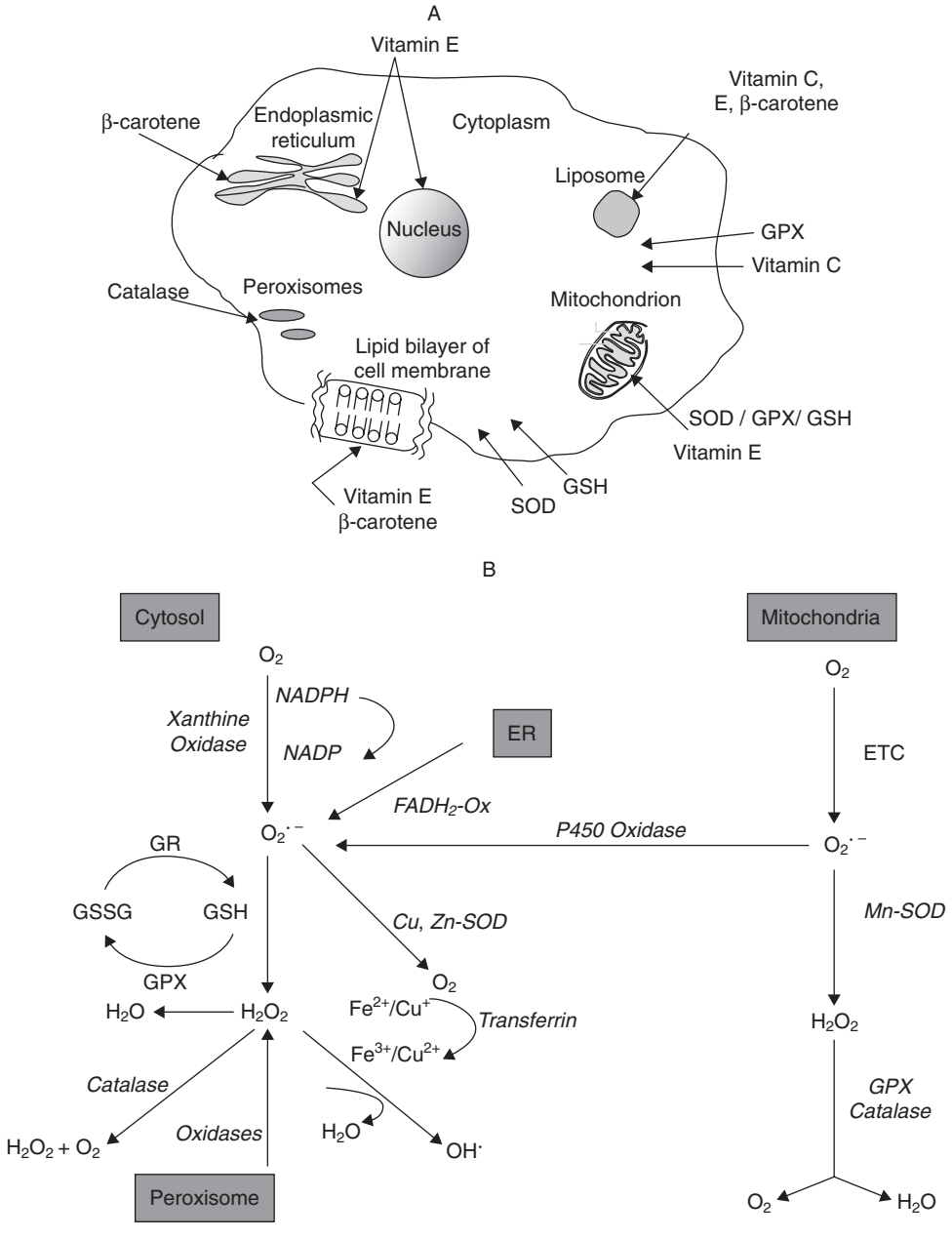

Figure 5.2. (A, B): Generation of ROS and endogenous antioxidant mechanisms. [ER: endoplasmic reticulum; ETC: electron transport chain; Ox: Oxidases] Redrawn and adapted from Wilcox 2002).

contains selenium. Polyphenols, especially flavonoids, have gained increasing interest because of their numerous biological effects such as free-radical scavenging, modulation of enzyme activities, inhibition of cell proliferation, as well as their potential utility as antibiotic, antiallergic, and anti-inflammatory agents (Bravo, 1998). They may be involved in the prevention of cardiovascular diseases, cancers, and other degenerative diseases (Scalbert et al., 2005). Due to their wide distribution in foods and beverages of plant origin such as fruit, vegetables, cereals, tea, coffee, cocoa, wine, and fruit juice, polyphenols can be considered as common micronutrients in human diet. Total polyphenol intake may vary depending on the diet. This daily intake is mainly constituted by hydroxycinnamates and flavonoids, which account respectively for about one-third and two-thirds of the total intake (Manach et al., 2004). It has been reported that the level of intake of flavonoids from diet is considerably high as compared to those of vitamin C (70 mg/day), vitamin E (7–10 mg/day), and carotenoids (β-carotene: 2–3 mg/day) (Yamasaki et al., 1997). The variations in consumption of foods and beverages are mainly responsible for the overall flavonoid intake in different national diets.

5.2.3 Antioxidant properties of flavonoids

A major role of flavonoids in both plants and animals is the protection they provide against oxidative stress. Flavonoids have been reported to scavenge free radicals such as hydroxyl ($^{\bullet}OH$), superoxide anion radical ($O_2^{\bullet -}$), and lipid peroxide radicals ($LOO^{\bullet}$) (Cotelle et al., 1996). Since oxidative stress plays a major role in the development of chronic diseases (Ames et al., 1993), it is believed that the consumption of polyphenol-rich foods may reduce the incidence and mortality rates due to chronic diseases. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase (Hanasaki et al., 1994). Flavonoids have been shown to inhibit cyclooxygenase, lipoxygenase, glutathione-*S*-transferase, and NADH oxidase, all involved in the generation of ROS (Kandaswami and Middleton, 1994). Many flavonoids can efficiently chelate trace metals such as iron and copper which are potential enhancers of ROS formation. For instance, hydrogen peroxide can be easily reduced to highly aggressive hydroxyl radical in the presence of iron:

$$H_2O_2 + Fe^{2+}\ (Cu^{+}) \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}\ (Cu^{2+})$$

Similarly, copper can mediate the oxidation of low-density lipoprotein (LDL) as below:

$$LH + L^{\bullet} \rightarrow LOO^{\bullet} \quad \text{(where LH represents LDL)}$$

Furthermore, the regeneration of α-tocopherol through the reduction of the α-tocopheroxyl radical may contribute to the antioxidant activity of flavonoids (Rice-Evans, 1995). In their radical scavenging reaction, a hydrogen atom is donated from the flavonoid to the attacking radical (Fig. 5.3a,b). R° represents superoxide anion, hydroxyl, peroxyl, or alkoxyl radical. Thus, a flavonoid free radical is formed which is more stable than the radical R° that generates it. Generally, the higher the number of OH substitution on the benzopyran ring, the stronger will be the radical scavenging activity of a flavonoid. Methylation of the free hydroxyl groups, therefore, may reduce antioxidant activity. Additional structural characteristics including a catechol or pyrogallol group in ring B, the combination of a double bond at C_2-C_3 and a OH group at C_3, or an oxonium ion (O^{+}) on

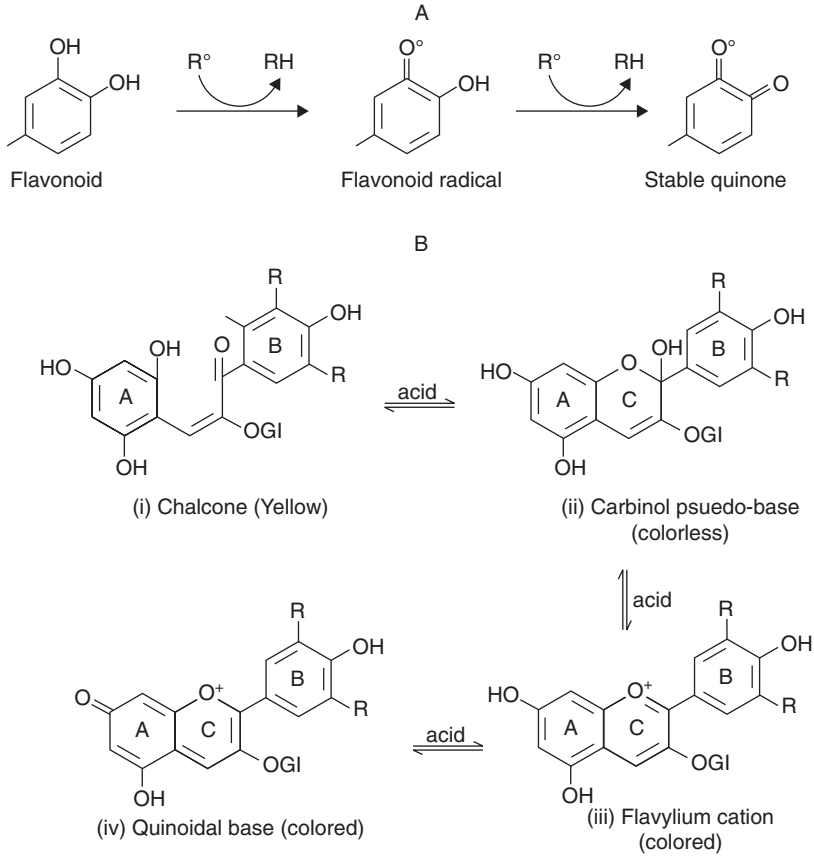

Figure 5.3. (A, B): Oxidation of an ortho-dihydroxy moiety of flavonoid by a reactive free radical resulting in the formation of a semiquinone and quinine. R° represents a free radical, RH is a reduced form (A). pH dependent changes in the protonation of anthocyanins giving rise to structures with different spectral (absorption) characteristics (B).

ring C, appear to enhance the radical scavenging activity, although the experimental data available are not conclusive at each point (Cao et al., 1997; van Acker et al., 1996; Cotelle et al., 1996).

5.3 ANTIOXIDANT ACTIVITY OF ANTHOCYANINS

Apart from their coloring effects in fruits, anthocyanins show ability to prevent lipid peroxidation in different lipid environments such as biomembranes and lipids bound to human LDL, as well as *in vitro* scavenging activity against various artificially generated free radicals (Rice-Evans et al., 2005; Andersen and Jordheim, 2006). Anthocyanins may exist in various protonated, deprotonated, hydrated, and isomeric forms, and the relative proportion of these molecules is strongly dependent on pH (Fig. 5.3b). The red flavylium cation is dominant at very acidic pH (pH 1–3). In aqueous media, as the pH is raised to a range of 4–5, hydration reactions generate the colorless carbinol pseudo-base, which can undergo

ring opening to the light yellow chalcones, most rapidly at pH 2.5–5 at increased temperatures. The flavylium cation can be transformed to quinoidal bases through a loss of protons and formation of quinine structure. These forms may play an important stabilizing role in the antioxidant action of anthocyanins (Dangles et al., 2000).

The completely conjugated structure of anthocyanins that allows electron delocalization results in very stable radical products, which is favorable considering their antioxidant ability (van Acker et al., 1996). The degree and position of hydroxylation and methoxylation in the B ring affect their stability and reactivity, and thereby, their antioxidant action (Rice-Evans et al., 2005). The hierarchy of antioxidant activities of fruits and vegetables studied showed that fruits that are rich in anthocyanins have a higher antioxidant potential than those rich in flavonones, flavanols, and hydroxycinnamates. When antioxidant activity of different phenolic fractions separated from Italian red wine were compared, it was found that anthocyanin fraction was the most effective in scavenging ROS and in inhibiting lipoprotein oxidation and platelet aggregation (Ghisseli et al., 1998).

5.4 ANTHOCYANIN BIOSYNTHESIS AND LOCALIZATION

Macromolecular organization of food involves several types of chemical interactions between food monomers and polymers manifesting into cells and tissues. Food microstructure is the organization of elements within a food and their interactions. Knowledge about the microstructure of foods is vital to control the properties of foods because there is a causal connection between structure and functionality (Aguilera et al., 2000). The microstructural elements in plant-derived foods include cell walls, starch granules, proteins, water and oil droplets, fat crystals, gas bubbles, and so forth. The nutrients found in the natural cellular compartments or within the assemblies produced during processing need to be released prior to or during digestion for their efficient absorption in the intestine. Nutrient absorption depends not only on the complete disruption of the cellular structure but also on the presence of and interaction with other food components. In addition, the physical state of the food matrix plays a key role in the release, mass transfer, accessibility, and biochemical stability of many food components (Aguilera, 2005). Bioavailability of phytochemicals from almond seeds was highly influenced by the degree of disruption achieved during consumption (Ellis et al., 2004). Disruption of the seeds either mechanically or by chewing resulted in the disruption of the first layer of cells adjacent to the fracture face, which minimized lipid release. Intact cells passed through the gut undigested and were excreted. However, if the cell walls were degraded by colonic fermentation, the lipids enclosed in the cell could be released. Thus, the food microstructure is an important factor in the release and bioavailability of several nutrients and has enormous consequences in assessing the nutraceutical properties of foods used in the prevention and therapy of some chronic degenerative diseases (Parada and Aguilera, 2007).

Polyphenols are the most widely occurring phytochemicals with a considerable physiological and morphological importance in plants, and these secondary metabolites are thus an abundant source of nutraceuticals in our diet. They have been proposed and shown to play several roles in the prevention of degenerative diseases by acting as antiallergic, antimicrobial, anti-inflammatory, antiatherogenic, antioxidant, cardioprotective, and vasodilatory agents. Grapes are rich sources of phenolic compounds such as flavan-3-ols (catechins), anthocyanins (in red grapes), and flavonols (Waterhouse and Walzem, 2005). Anthocyanins are water-soluble pigments responsible for all the color differences between grapes and the

resulting wines. They have also been associated with the health benefits derived from consuming high levels of fruits, vegetables, and wine. However, the bioavailability of anthocyanins is estimated to be very low, and their metabolism is still not fully understood. Because a major part of the polyphenols ingested (75–99%) is not found in urine, they may have been either not absorbed through the intestinal barrier, absorbed and excreted in the bile, or metabolized by colonic microflora (Parada and Aguilera, 2007). McDougall et al. reported that polyphenols are transiently bound to food matrices during digestion and that this protects the more labile anthocyanins from degradation (McDougall et al., 2005).

In most plants, anthocyanins are found uniformly dissolved in the vacuolar solution of epidermal cells. However, in certain species, they are found localized in discrete regions of the cell vacuole (Markham et al., 2000). Such pigmented bodies, which are membrane-bound organelles that provide an intense coloration in the vacuoles of mature plant cells, have been described by Pecket and Small (1980) as anthocyanoplasts. Later, these globular inclusions have been reported as protein matrices (Nozue et al., 1995, 1997) without a membrane boundary or an internal structure (Nozzolillo, 1994; Cormier, 1997). These insoluble protein matrices, called anthocyanin vacuolar inclusions (AVIs), are thought to sequester anthocyanins, thereby increasing their stability and reducing the inhibition of certain vacuolar enzymes (Markham et al., 2000). Protoplasts of anthocyanin-containing grapevine cells (*Vitis vinifera* cv. Gamay) (Calderón et al., 1993) contained anthocyanin-containing vesicles (anthocyanoplasts), whereas no anthocyanoplasts were found in isolated vacuoles, and it was concluded that anthocyanoplasts are mainly located in the cytoplasm. Similar studies using a grapevine cell suspension culture implied the vacuolar localization of numerous small pigmented bodies (1–3 μm in diameter), which presumably coalesced inside the vacuole to form a single large body up to 15 μm in diameter. These bodies freely moved around the majority of the cell by Brownian motion on unfixed samples, being retarded only by the inner face of a visible membrane. Furthermore, when released into the buffer, they remained intact for a short time with no apparent delimiting membrane. This evidence indicated their nature as macromolecular complexes of vacuolar localization (Conn et al., 2003). A new insight into the structure and formation of AVIs in flower petals was obtained by using light and electron microscopy (EM) (Zhang et al., 2006). Investigations on AVIs in the epidermal cells of different regions of lisianthus flower petals revealed three different forms of AVIs: vesicle-like, rod-like, and irregular shaped. Electron microscope examinations showed no membrane encompassing the AVI; instead, the AVI itself consisted of membranous and thread-like structures throughout. It was also found that anthocyanins accumulated as vesicle-like bodies in the cytoplasm and were contained in the prevacuolar compartments (PVCs). These vesicle-like bodies were transported into the central vacuole by merging of the PVCs and the central vacuole in the epidermal cells. The anthocyanin-containing vesicle-like bodies were subsequently ruptured to form threads in the vacuole.

Polyphenols are nutritionally important components that provide health beneficial properties to fruits and its processed products (Clifford and Brown, 2006). Fruit juice is an important medium for obtaining polyphenols, soluble and insoluble fibers, vitamins, and various other components. Fruit juices can be either whole fruit blend, such as tomato and citrus, or clarified juices, such as grape and various berries. During homogenization of fruits and vegetables for juice processing, the structural and functional components may be further modified influencing the microstructure of the juice and potentially affecting its properties, thus influencing the bioaccessibility and bioavailability of nutraceutical ingredients.

5.5 BIOACCESSIBILITY AND BIOAVAILABILITY OF POLYPHENOLS

The level of bioactive ingredients absorbed into the body is affected by their bioaccessibility and bioavailability. Bioaccessibility refers to the amount of potentially absorbable form of the active ingredient from the GIT. Bioavailability refers to the amount of active ingredients actually absorbed into the body. The bioavailability in turn is tremendously influenced by the bioaccessibility. The bioaccessibility of active ingredients is influenced by the structure of the molecule and its complex formation with the food matrix (Holst and Williamson, 2008). A large number of studies using *in vivo* models suggest that absorption of flavonoids (e.g., quercetin, quercetin glucoside, catechins including tea catechins) is tremendously influenced by the food matrix interactions. The type of sugar attached to the flavonoid moiety, the presence of lipids in the diet, the presence of emulsifiers, and other co-ingested food ingredients such as carbohydrates, fiber, and alcohol, in general enhanced the uptake of flavonols, flavanols, flavanones, and isoflavones (Scholz and Williamson, 2007). Polyphenols such as anthocyanins may exist in a free as well as complexed state because of the deteriorative changes that occur in ripening fruits that release lipid vesicles, polysaccharide molecules such as pectin and cell wall components. During processing of fruits which involves homogenization of the tissue, decompartmentalization of various ingredients and complex formation are enhanced. Thus, several physiological and physical factors influence the bioaccessibility and bioavailability of active ingredients.

According to Lipinski's rule of five (Lipinski et al., 2001), orally ingested components with molecular masses >500, more than 5 hydrogen bond donors and 10 hydrogen bond acceptors, and high lipophilicity (octanol : water partition coefficient cLog $P > 5$) are likely to be poorly absorbed. Anthocyanins usually exist as their glycosides thatprovide a high degree of hydrogen bonding capability with the food matrix such as proteins and carbohydrates. Biotransformations involving catabolic breakdown, methylation, deglycosylation, and so on, have been suggested to enhance bioavailability (Yang et al., 2008). The differences in the efficacies between *in vitro* and *in vivo* studies may be due to such variabilities. During *in vitro* studies, cell lines of different physiological origin are challenged with bioactive components that these cells may not encounter naturally, and these components may be taken up by the cells. Under *in vivo* conditions, these bioactive components may be metabolized and absorbed, and their efficacy could be different. Thus, studies are needed to clearly understand the factors that would enhance the bioavailability of anthocyanin components from fruits so that their *in vitro* efficacies could effectively be reproduced after consumption of fruits or fruit products, and translated into prevention of chronic degenerative diseases.

Several reviews summarize the studies on the bioaccessibility and bioavailability of polyphenols including anthocyanins in mammalian systems (Manach et al., 2004, 2005; Scholz and Williamson, 2007; Holst and Williamson, 2008; Vitaglione et al., 2008; Yang et al., 2008). Due to their instability at higher pH values, the analysis of anthocyanins in body fluids is difficult, but absorption and excretion of very low amounts of the intact glycosides have been reported after ingestion of anthocyanin-rich berry extract or wine. In a study involving five healthy human volunteers, 200 g blackberries (960 μmol of anthocyanin) were fed, and urine samples were analyzed. In addition to native cyanidin-3-glucoside, several other anthocyanin metabolites were identified in the urine: methylated glycosides, glucuronides of anthocyanidins and anthocyanins, a sulfoconjugate of cyanidin, and anthocyanidins (Felgines et al., 2005). Wu et al. (2002) studied the absorption and

metabolism of anthocyanins in four elderly women fed with 12 g elderberry extract (720 mg total anthocyanin) and six elderly women fed with 189 g lowbush blueberry (690 mg total anthocyanin). Two major anthocyanins in elderberry extract, viz., cyanidin-3-glucoside and cyanidin-3-sambubioside, together with four metabolites, peonidin-3-glucoside, peonidin-3-sambubioside, peonidin-monoglucuronide, and cyanidin-3-glucoside monoglucuronide, were identified in urine within 4 hours of ingestion. The average elderberry anthocyanin excretion was 554 μg (0.08% intake in 4 hours). Urine samples of women fed with blueberry retained anthocyanins in the original form and the total urinary excretion during the first 6 hours was 23 μg. Plasma anthocyanin levels were below detection limit demonstrating low absorption of anthocyanins. Talavera et al. (2005) measured anthocyanin distribution in the stomach, jejunum, liver, and kidney, as well as the brain in rats fed with a blackberry anthocyanin-enriched diet for 15 days. The stomach tissue contained only native blackberry anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-pentoside), while in jejunum, liver, and kidney, native and methylated anthocyanins as well as conjugated anthocyanidins (cyanidin and peonidin monoglucuronides) were identified. Proportions of anthocyanin derivatives differed according to the organ analyzed, with the liver having the highest proportion of methylated forms. This study demonstrated the presence of the aglycone form in the jejunum and plasma. In the brain, total anthocyanin content (blackberry anthocyanins and peonidin 3-*O*-glucoside) reached 0.25 nmol/g of tissue. Additional studies on the concentration of flavonoids and their metabolites are needed to identify target tissues for the various flavonoids, which may help understand their mechanisms of action *in vivo* (Williamson and Clifford, 2010).

5.6 MICROSTRUCTURAL CHARACTERISTICS OF GRAPE JUICE

Microstructure of grape juice was examined to understand the nature of interactions between anthocyanins and food matrix. Fruits have a heterogeneous assembly of macromolecular structures, such as cell wall components comprising cellulose and pectin, various types of membranes that provide compartmentalization, and simple chemical components that provide several qualities such as color, taste, and flavor. The ripening process involves the controlled deterioration of the structural components. Cellulose and pectin are broken down into smaller units to form soluble fiber components. Starch or organic acids are converted into sugars. The membrane phospholipids are catabolized into neutral lipids such as diacylglycerols and free fatty acids. The accumulation of degradation products in the membrane results in the microvesiculation of the membrane. Thus, a ripe fruit provides a variety of components that impart nutritional quality in either the fresh or the processed form (Oke and Paliyath, 2006a,b).

The method of juice preparation may affect the composition of the juice. For instance, hot break processing inhibits pectinolytic enzymes, resulting in the formation of large molecular-weight pectic polymers that give a thick consistency to tomato juice and sauce (Oke and Paliyath, 2006a,b). Commercially available grape juice concentrate is processed through several steps that involve homogenization, filtration, pasteurization, and concentration by heat or other methods. These techniques may also help to remove haze-forming components such as proteins and procyanidins (Bates et al., 2001). Exposure to such physical parameters can alter the composition and intermolecular interactions because the compartmentalization of the cells is disrupted, and several cell components (cell wall, membrane,

Table 5.1. Total polyphenol content in grape juice preparations

Sample	Total polyphenols
Grape juice concentrate	$627 \pm 85^{b,c}$
Dialyzed fraction of grape juice concentrate	$143 \pm 13^{a,c}$
Dialyzate of grape juice concentrate	$445 \pm 30^{a,b}$

[a] Denotes statistical significance at $P < 0.05$ from grape juice concentrate.
[b] Denotes significance from dialyzed fraction of grape juice concentrate.
[c] Denotes significance from dialyzate of grape juice concentrate.
The values are normalized to show the amount in µg in 1 mL of grape juice concentrate, 1 mL of dialyzed fraction of grape juice concentrate, and total amount in the dialyzate from 1 mL of concentrate.
Source: Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

polyphenols, flavor, organic acids, etc.) are free to interact with each other. Such interactions may alter the functionality of various ingredients from that observed for the free molecules. Essentially, juice concentrates may show properties that are quite different than those observed for the individual constituents. The complexity of the juice composition potentially suggests the existence of interactions between the various types of molecules. Such interactions may affect the nature of transit, stability, and bioavailability of key components such as polyphenols in the gastrointestinal system. The bioavailability of anthocyanins is in the range of 0.61–1.82% (Ichiyanagi et al., 2006), which is not very high when compared to the amount present in the juice or in fruits. This suggests that a good portion of polyphenols may escape absorption in the small intestine but are carried over to the large intestine, where they may exert an unknown physiological function or get metabolized and absorbed into the body. It is generally assumed that the food matrix in fruit juices may not exert an influence on the availability of polyphenols. But fruit juice processing may change the molecular interactions between various components, thus altering the structure, bioavailabilty, and physiological properties. Recent studies have suggested the existence of potential interactions between polyphenols and polysaccharides in wine (Saura-Calixto and Diaz-Rubio, 2007).

Dialysis of concentrated commercial grape juice against water results in a considerable portion of the anthocyanins being confined within the dialysis bag. If all the anthocyanins were free, the concentration of polyphenols, inside and outside the dialysis bag would have been expected to be equal. The polyphenol contents of the grape juice concentrate, the dialyzed fraction, and the dialyzate are shown in Table 5.1. The original concentrated grape juice contained about 627 µg/mL polyphenols. After dialysis using a 6–8 kDa molecular weight cutoff dialysis membrane, 445 µg or about 70% of the polyphenols were obtained in the 2-L dialyzate solution (significant at $P<0.05$). Nearly 143 µg or about 22% was recovered in the dialyzed juice. Thus, a statistically significant ($P<0.05$) amount of polyphenols were retained as nondialyzable complexes (Jacob and Paliyath, 2008).

5.7 PHYSICOCHEMICAL PROPERTIES OF THE DIALYZED JUICE FRACTION

The juice fraction obtained by dialysis of grape juice concentrate was subjected to various treatments to analyze the physicochemical properties of carbohydrate–polyphenol

Table 5.2. Effect of sucrose, Triton X-100, and digestive enzymes on the dialyzed fraction of grape juice concentrate. The dialyzed fraction was subjected to various treatments and again dialyzed to remove released polyphenols (except when treated with sucrose solution, where the solution was centrifuged at 105,000×g to pellet the bound polyphenols). The amount of polyphenols left in the dialyzed solution was estimated

Treatment	Concentration levels of treatments	Total polyphenol content (mg/L)
Sucrose	Control	$143 \pm 13^{b,c}$
	2.5%	$64 \pm 1\ 3^{a}$
	5.0%	67 ± 15^{a}
Triton X-100	Control	$143 \pm 13^{b,c,d}$
	0.005%	$115 \pm 32^{b,c}$
	0.01%	$94 \pm 25^{b,c}$
	0.02%	$71 \pm 25^{a,b,c}$
Digestive enzymes	Control	143 ± 13^{f}
	pH 2.0	129 ± 6
	Trypsin	136 ± 11
	Trypsin + pH 2.0	136 ± 13
	Pancreatic lipase	128 ± 10
	Pancreatic lipase + pH 8.0	111 ± 10^{a}

Statistically significant means are denoted by different superscripts.
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complexes. Treatment with sucrose resulted in the release of nearly 50% of the polyphenols bound to the matrix, potentially suggesting hydrogen bond interactions between polyphenols and the matrix (Table 5.2). Treatment with a detergent such as Triton X-100 also caused the release of polyphenols in a concentration-dependent manner, suggesting that polyphenols may be confined in lipid vesicular structures that would get solubilized and rupture in the presence of the detergent (Table 5.2). Digestive enzymes such as trypsin and lipase were also used to study the stability of the polyphenol microaggregates in the dialyzed fraction of grape juice concentrate. The pH was varied to simulate conditions that are normally encountered in the stomach. It was found that the total polyphenol content was unaffected at pH=2.0 or with trypsin (alone or at pH=2.0). With the lipase treatment at pH=8.0, the total polyphenol content dropped by nearly 24% in comparison to the control (significant at $P<0.05$), whereas the total polyphenol content was unaffected for the dialyzed juice treated with lipase alone (Table 5.2).

5.8 ULTRASTRUCTURAL ANALYSIS OF JUICE FRACTIONS

The retention of a significant amount of polyphenols inside the dialysis bag after extensive dialysis suggested that these may be complexed to materials of large molecular weight. To obtain a better structural understanding of such entities, electron microscopic analyses of the juice preparations were conducted. A brief staining with uranyl acetate provides positive staining to electron-dense materials attached to the fomvar-coated grids. A representative

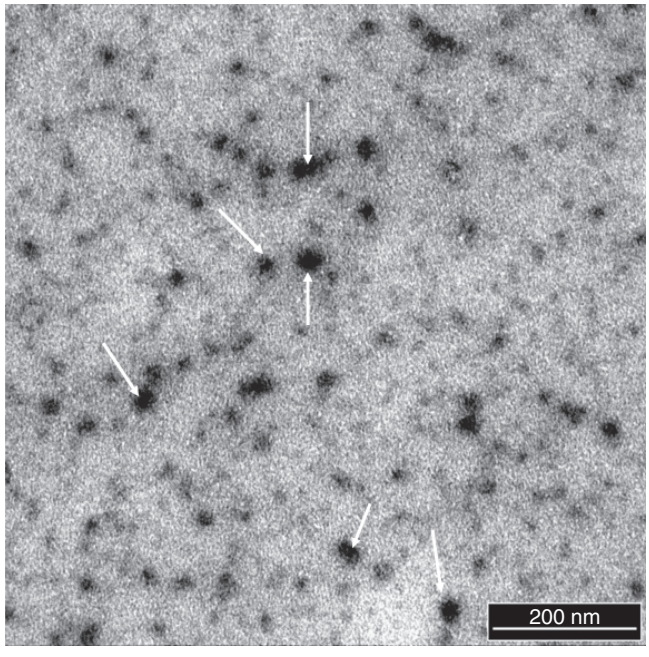

Figure 5.4. Transmission electron micrograph of pectin–polyphenol–lipid nanovesicle complexes in dialyzed grape juice concentrate as revealed by positive staining with uranyl acetate. The complexes are marked by white arrows. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

morphology of the macromolecular complexes from the dialyzed juice is shown in Figure 5.4. Dark staining areas varying in diameter from 10 to 100 nm were detectable (arrows). Many of the aggregates also revealed the presence of minute vesicular structures. Carbohydrate polymers by themselves do not stain with uranyl acetate; therefore, the visible structures must contain components such as polyphenols that would react with uranyl acetate. The treatment with Triton X-100 (Table 5.2) did not fully release the polyphenols, suggesting that a large proportion of polyphenols are bound potentially to carbohydrate polymers. A representative field of dialyzate subjected to the same analysis did not reveal any such structures, indicating the true nature of such macromolecular complexes in the dialyzed juice.

To determine the nature of potential carbohydrate moieties involved in the formation of macromolecular complexes, the dialyzed juice was subjected to treatments with carbohydrate-degrading enzymes. Since grapes are primarily acidic and rarely contain starch after ripening, amylases that catabolize starch were not expected to have a significant effect on the complexes. Treating the dialyzed juice with cellulase did not result in major changes in the structure of the complexes. However, enzymes that degrade pectin, such as pectinase and β-galactosidase, caused a disruption of the macromolecular complexes, suggesting that polyphenols that are confined in vesicles are associated with pectin matrix (Fig. 5.5).

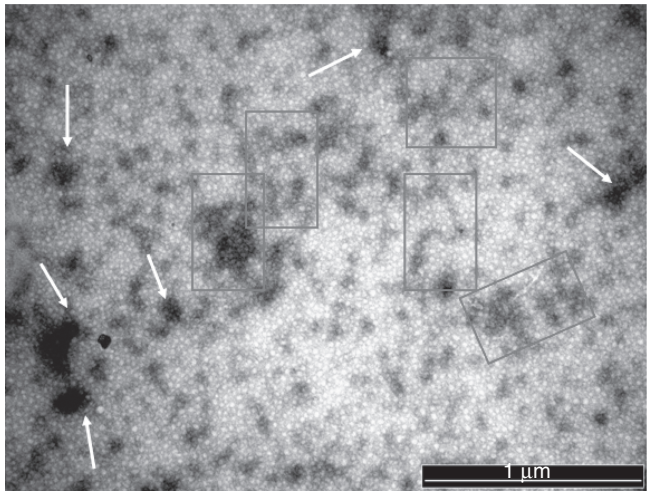

Figure 5.5. Transmission electron micrograph of macromolecular complexes and vesicles in dialyzed juice subjected to treatment with pectinase and further subjected to dialysis before EM. The grid was stained with uranyl acetate. The undigested complexes are marked by white arrows. Regions showing vesicular aggregates are shown by rectangular boxes. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

5.9 COMPOSITION OF JUICE FRACTIONS

Further evaluation of food matrix–polyphenol interaction was conducted by quantifying potential differences in the polyphenol composition of the grape juice concentrate, the dialyzed juice, and the dialyzate. The polyphenols were isolated from these juice fractions by hydrophobic interaction chromatography, and equal amounts were subjected to high-performance liquid chromatography–mass spectrometry (HPLC-MS). The polyphenol composition of various juice fractions are shown in Table 5.3. There were major differences in the polyphenol compositions of the three samples. The dialyzed fraction contained -3-*O*-diglucosides of cyanidin and delphinidin that were barely detectable in the original juice and dialyzate. -3-*O*-glucosides of delphinidin, cyanidin, and malvidin were present in the juice and the dialyzed juice. The dialyzate showed significantly higher levels of cyanidin-3-*O*-glucoside ($P<0.05$). Malonyl glucoside of malvidin was at detectable levels only in the dialyzed juice. The -3-*O*-acetoyl glucosides of delphinidin, petunidin, and malvidin were nearly evenly distributed in the polyphenol fraction of the concentrated juice and dialyzate but at a lower level (~50%) in the polyphenols of the dialyzed fraction (significantly different at $P<0.05$). The polyphenols from the dialyzed fraction also showed the presence of feruloyl and coumaroyl glucosides of cyanidin. The polyphenols from the concentrated juice contained a significantly higher proportion of coumaroyl glucosides of delphinidin and petunidin than those of the dialyzed fraction. Interestingly, the polyphenols from the dialyzed fraction also contained nearly double the amount of coumaroyl glucoside of malvidin in comparison to the polyphenols of the juice concentrate or the dialyzate (significantly different at $P<0.05$). The macromolecular complexes perhaps may provide better conditions of interactions for relatively more hydrophobic polyphenols. Thus, there may be specific interactions between polyphenols and food matrix.

Table 5.3. LC-MS profile of polyphenols present in grape juice concentrate, its dialyzed fraction, and the dialyzate (Mean ± SE from three independent estimations)

Anthocyanins identified by LCMS	**Anthocyanin (% wt basis)**		
	Grape juice concentrate	**Dialyzed fraction**	**Dialyzate**
Cyanidin-3-O-diglucoside	BDL	0.30 ± 0.01	BDL
Delphinidin-3-O-diglucoside	BDL	5.72 ± 0.1	BDL
Delphinidin-3-O-G	9.83 ± 1.8	BDL	10.57 ± 0.7
Cyanidin-3-O-G	6.02 ± 2.0^{c}	BDL	9.19 ± 0.2^{a}
Petunidin-3-O-G	Traces	BDL	Traces
Malvidin-3-O-G	3.42 ± 1.8	4.43 ± 0.01	5.24 ± 0.8
Malvidin-3-O-malonyl G	BDL	2.52 ± 0.2	BDL
Delphinidin-3-O-acetoyl G	3.29 ± 0.9^{c}	2.80 ± 0.1^{c}	5.94 ± 0.5^{a,b}
Petunidin-3-O-acetoyl G	6.73 ± 2.4^{b}	3.27 ± 0.1^{a,c}	8.42 ± 1.9^{b}
Malvidin-3-O-acetoyl G	9.58 ± 1.4^{b}	3.65 ± 0.01^{a,c}	7.31 ± 2.1^{b}
Cyanidin-3-O-feruloyl diglucoside	BDL	2.47 ± 0.9	BDL
Cyanidin-3-(6′-coumaroyl) G	BDL	3.09 ± 0.02	BDL
Delphinidin-3-(6′-coumaroyl) G	12.43 ± 0.3^{c}	BDL	8.32 ± 2.3^{a}
Petunidin-3-(6′-coumaroyl) G	10.28 ± 2.2^{b,c}	2.50 ± 0.01^{a}	4.06 ± 0.4^{a}
Malvidin-3-(6′-coumaroyl) G	38.41 ± 8.2^{b}	69.34 ± 3.6^{a,c}	40.96 ± 8.0^{b}

Different superscripts a, b, and c denote statistical significance at $P<0.05$ for grape juice concentrate, dialyzed fraction of grape juice concentrate, and the dialyzate, respectively.
Source: Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.
G: glucoside; BDL: below detection limit.

Proton nuclear magnetic resonance (NMR) analyses of various juice fractions were conducted to understand the chemical nature of the macromolecular components (Jacob and Paliyath, 2008). The NMR spectrum of pure malvidin-3-*O*-galactoside chloride showed resonances characteristic of the A, B, and C ring protons and the glucose moiety (Fig. 5.1). The spectrum shows well-resolved resonances from the aromatic and aliphatic sugar protons, spanning from 6 to 8.4 ppm and from 3.5 to 4 ppm, respectively (Fig. 5.6). There are two potential anomeric duplets appearing at 4 and 5.15 ppm. The 3′ and 5′ positions of malvidin are methylated, and the signals from the two -O-CH3 groups appear at 3.75 ppm as sharp singlets. The signals from the 2′ and 6′ protons appear at 6.95 and 7.25 ppm as duplets (designated as 2). The signal from the proton at position 4 of the B ring is potentially the most shielded and was projected to appear at 8.8 ppm as a duplet (peak designated as 1). The signals from the protons at C6 and C8 of the A ring are projected to appear at 6.25 and 6.35 ppm (group designated as 3). In general, the spectrum of malvidin-3-*O*-galactoside chloride is very similar to that of other anthocyanins but with fewer aromatic protons.

The 1H NMR spectrum of the dialyzed grape juice was acquired under the exact same conditions as those employed for authentic malvidin-3-O-galactoside and is shown in Figure 5.7. An interesting aspect of this spectrum is the near disappearance of the protons in the aromatic region, potentially because of molecular interactions and broadening. Such a region is designated by 1 in the spectrum. Because of the complex nature of carbohydrates, the carbohydrate region of the signals appears to range from 3.5 to 5.5 ppm with multiple peaks and a broad multiplet (peaks designated by 2, 3, 5, and 6). Peak 4 is a remnant of the suppressed water protons. Additional signals are observed at 1.2 ppm, potentially indicating the presence of methylene and methyl protons arising from lipid

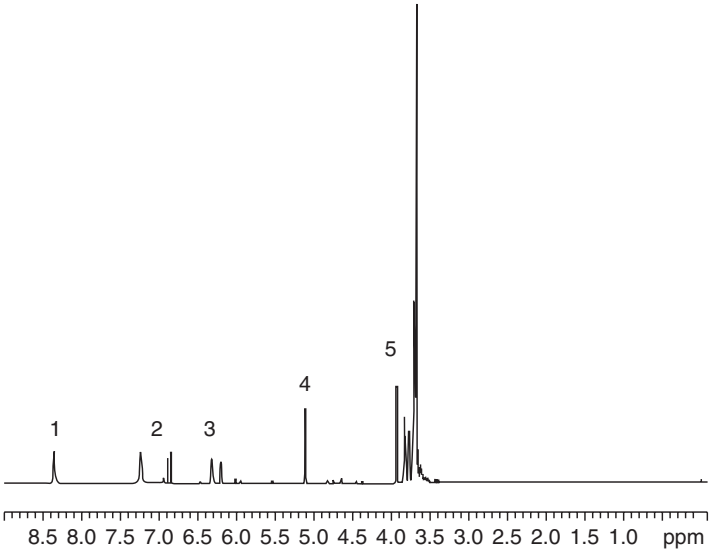

Figure 5.6. ^{1}H NMR spectrum of malvidin-3-*O*-galactoside chloride recorded in deuterated water. Peaks at 1, 2, and 3 correspond to protons in the aromatic region. Peak 4 is unidentified. The signals from galactose occur between 3.5 and 5 ppm. The sharp singlet at 3.7 ppm corresponds to the -*O*-methyl resonance from the B ring. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

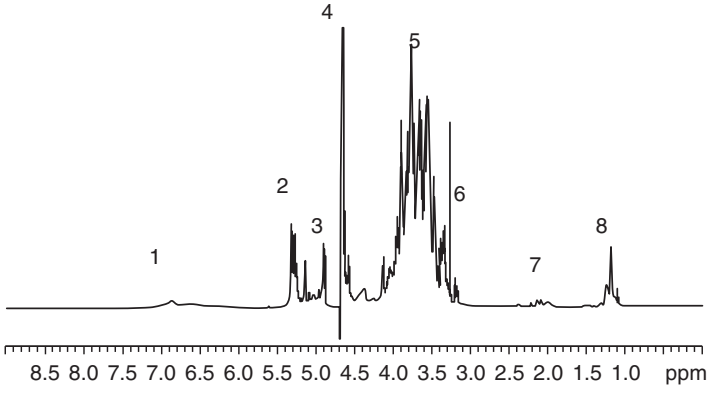

Figure 5.7. ^{1}H NMR spectrum of dialyzed juice recorded in deuterated water. Broad peaks at 1 may correspond to aromatic protons. Peak 4 is the suppressed water signal. Peaks 2, 3, and 5 originate from carbohydrate protons. Peak 6 may originate from an -*O*-methyl group which is distinct from the sharp singlet at 3.7 ppm. Peak 8 corresponds to lipid methylene protons. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

vesicles (peaks designated as 8). The signals arising at 2.2 ppm (designated as 7) are unknown (Fig. 5.7).

Pectinase treatment of the dialyzed juice resulted in a significant reduction of carbohydrate resonances (Fig. 5.8) appearing between 3 ppm and 5.2 ppm (peaks designated 1, 3, 4). Peak 2 is a suppressed water proton signal at 4.8 ppm. The signals which appeared

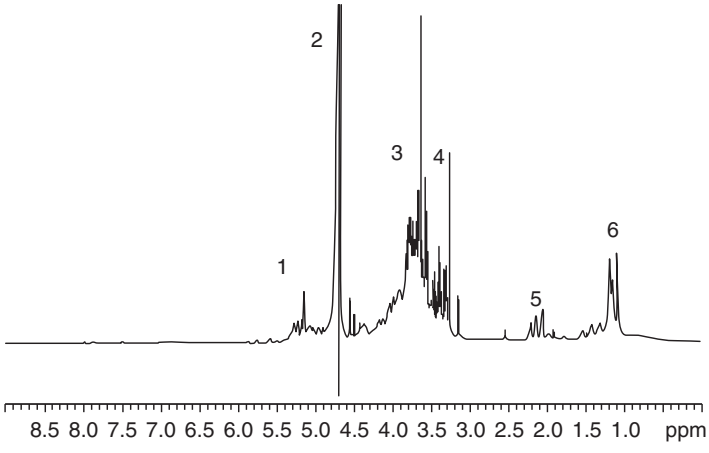

Figure 5.8. ^{1}H NMR spectrum of pectinase-treated dialyzed juice recorded in deuterated water. Broad peaks at 1 appear to have been reduced in intensity after pectinase treatment. Peak 2 originates from water. The carbohydrate proton signals (3–4.5 ppm) appear to be reduced in intensity. The sharp singlets in this group may originate from -*O*-methyl protons. Peaks at 5 are unidentified. Peaks at 6 may originate from lipid methylene (broad) and methyl protons (CH3-CH2, triplet). Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

upstream of 4 ppm in the carbohydrate signals of dialyzed juice (Fig. 5.7, peaks 2,3) appears to have been considerably reduced, indicating them to be polygalacturonic acid-specific protons. As well, the sharp singlets among the carbohydrate signals may arise from -O-CH_3 groups of anthocyanins and pectin. The signals from the lipid methylene protons (group designated as 6) appearing at 1.2 ppm appear to have increased after pectinase treatment. The resonance signals from unknown components (group designated as 5) also showed an increase in intensity after pectinase treatment.

^{1}H-NMR spectrum of the concentrated polyphenols in the dialyzate (Fig. 5.9) shows a complex group of signals (designated as 1–6). Interestingly, the signals from the aromatic protons appear to be missing or masked. The carbohydrate region between 3 and 4 ppm is much simplified. The triplet at 1 ppm (peak 6) is suggestive of a -CH2-CH3 group. The signals from the anthocyanins appear to be totally different from those characteristic of pure anthocyanins, potentially indicating complex formations or structural alterations.

5.10 ANTIOXIDANT ACTIVITY OF JUICE FRACTIONS

The functional properties of polyphenols may be affected by complex formation. The ability of the polyphenols in concentrated grape juice, the dialyzed juice fraction, and the dialyzate to scavenge superoxide, hydroxyl, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was quite different (Jacob and Paliyath, 2008) (Fig. 5.10a–c). Polyphenols have a very strong antioxidant activity that has been attributed to their structure (Cook and Samman, 1996). The most efficient antioxidant activity is observed when the structure shows unsaturation between carbons 2 and 3 (C2-C3) of the C ring and in the presence

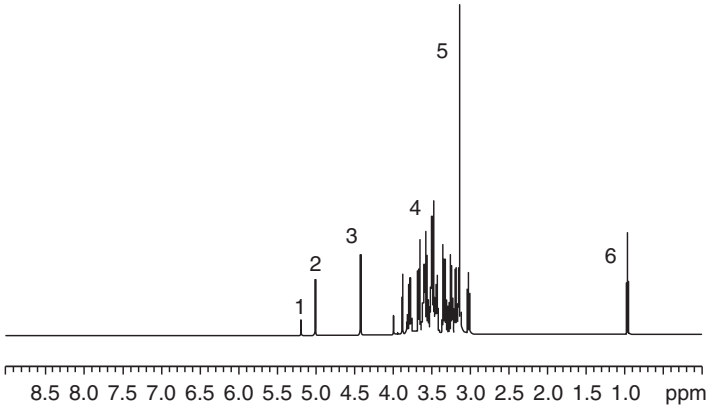

Figure 5.9. ^{1}H NMR spectrum of the dialyzate containing polyphenols recorded in deuterated water. The aromatic proton signals are missing. Peaks 1–4 may originate from various sugar moieties of polyphenols. Peak 5 at 3.2 ppm may originate from -O-methyl groups. Peak 6 corresponds to a methyl group with an adjacent CH2. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

of a free hydroxyl group at position 3 (3-OH) of the C ring. Antioxidant activity has been measured by using several techniques such as DPPH, ferric ion reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and so forth (Sanchez-Moreno, 2002). To evaluate the influence of complex formation on antioxidant activity, superoxide-, hydroxyl-, and DPPH-radical scavenging capacities of the polyphenols isolated from the original juice concentrate, the dialyzed juice, and the dialyzate were monitored. It was interesting to note that the superoxide scavenging property of the polyphenols from the dialyzed juice was undetectable, whereas dose-dependent increases in activities were observed in the polyphenols from the juice concentrate and the dialyzate (Fig. 5.10a). A lower magnitude of scavenging was observed for hydroxyl radicals by polyphenols from the dialyzed fraction (Fig. 5.10b). All the fractions showed a similar scavenging potential toward DPPH radicals (Fig. 5.10c). These results may suggest the occurrence of structural modifications in at least some proportion of the polyphenols during processing that may alter their ability to scavenge the free radicals. This may be due to complex formations involving the key hydroxyl groups, making them less amenable to the exchange of protons. Thus, conventional processing may reduce some of the key functionalities of polyphenols. Improvements in processing technology may have to be adopted to help preserve the nutritional quality of fruit juice. As discussed earlier, the homogenization, extraction, and concentration steps would have facilitated the association of different cellular macromolecules into well-defined structures that may affect the food properties and functionality of ingredients associated with such structures. In a recent study (Saura-Calixto and Diaz-Rubio, 2007), it was reported that nearly 35% of the polyphenols in red wine is bound with soluble dietary fiber and is not accessible for analysis. It was also proposed that such polyphenol–soluble fiber complexes may pass through the intestine without the polyphenols getting absorbed and reach the colon. Colonic fermentation may further release these polyphenols and provide an antioxidant environment in the colon. Despite the loss in some functional aspects, the polyphenol–carbohydrate–lipid complexes may play some hitherto unidentified functional role in the human diet.

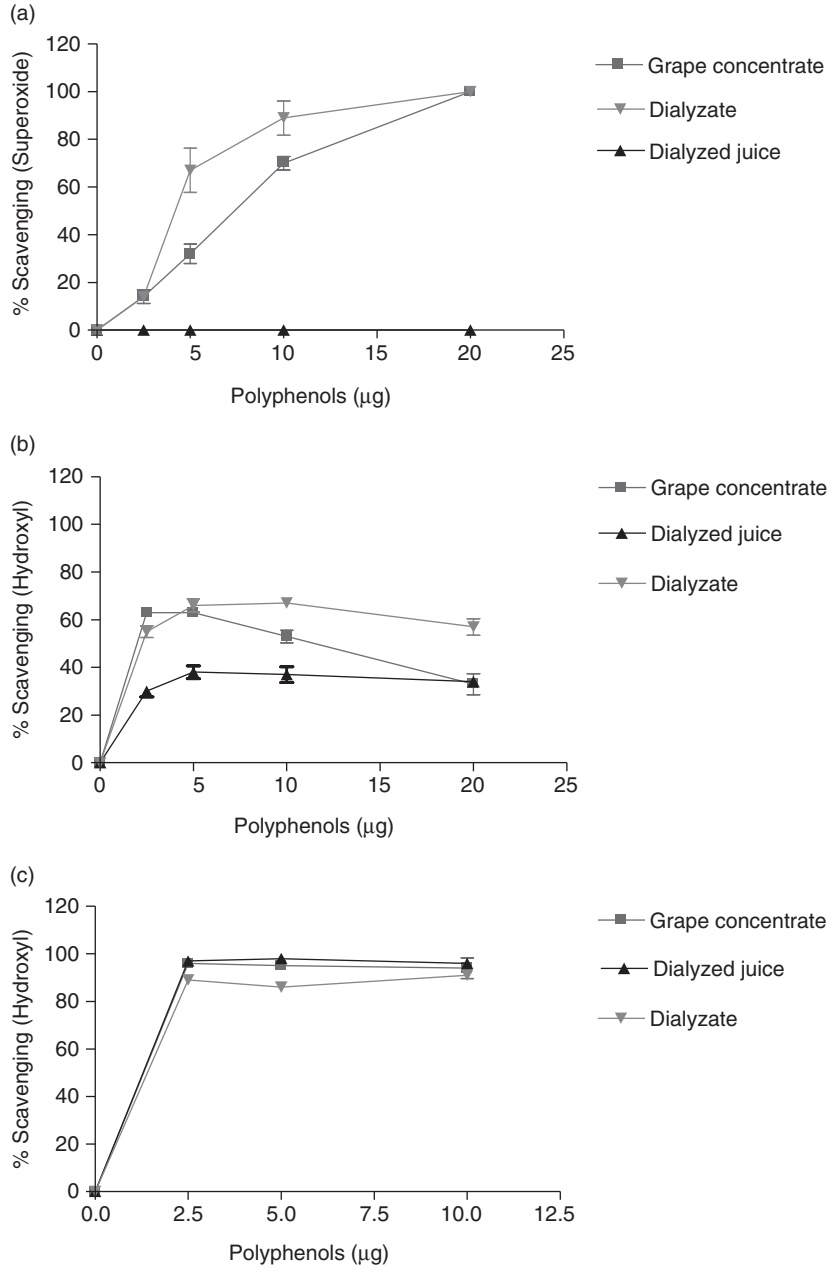

Figure 5.10. (A) Superoxide scavenging efficiency of the polyphenols from grape juice concentrate, dialyzed juice, and the dialyzate; (B) hydroxyl radical scavenging efficiency of polyphenols isolated from grape juice concentrate, dialyzed juice, and dialyzate; and (C) DPPH radical scavenging efficiency of polyphenols isolated from grape juice concentrate, dialyzed juice, and dialyzate. Reproduced with permission from Jacob and Paliyath, 2008, *Journal of Agricultural and Food Chemistry*, 56, 1305–1315.

5.11 METABOLISM AND BIOAVAILABILITY OF FLAVONOIDS

Despite the increasing evidence for the *in vitro* antioxidant potential of flavonoids, little is known about their efficacy *in vivo*, and this may be ascribed to the limited knowledge on their bioavailability in humans. The extent of absorption of dietary polyphenols in the small intestine is relatively small (Spencer and Rice-Evans, 2003). Unabsorbed flavonoids, and those absorbed, metabolized in the small intestine and liver, and transported back into the intestinal lumen, ultimately reach the large intestine where they are further metabolized by gut microflora to smaller phenolic acids (Fig. 5.11). Bacterial enzymes may catalyze several reactions such as hydrolysis, dehydroxylation, demethylation, ring cleavage, and decarboxylation. Using rat models, de Boer et al. (2005) reported that after 11 weeks of feeding quercetin, its metabolites were identified in many rat tissues, with the highest levels found in the lungs, and the lowest in the brain, white fat, and spleen. Several studies have reported the presence of various flavonoids such as naringenin, tangeretin, genistein, epicatechin, and anthocyanins in the brain tissue of rats (Andres-Lacuevea et al., 2005; Talavera et al., 2005). Anthocyanins (cyanidin-3-galactoside, cyanidin-3-arabinoside, malvidin-3-glucoside, malvidin-3-galactoside, malvidin-3-arabinoside, peonidin-3-arabinoside, delphinidin-3-galactoside) were found in the cerebellum, cortex, hippocampus, and stratum of rats fed with blueberries for 10 weeks. This indicates that flavonoids can cross the blood–brain barrier and localize in various brain regions important for learning and memory (Andres-Lacuevea et al., 2005). Paganga and Rice-Evans (1997) reported the absorption of flavonoids and their presence in human plasma in the glycosylated form. Rutin and other

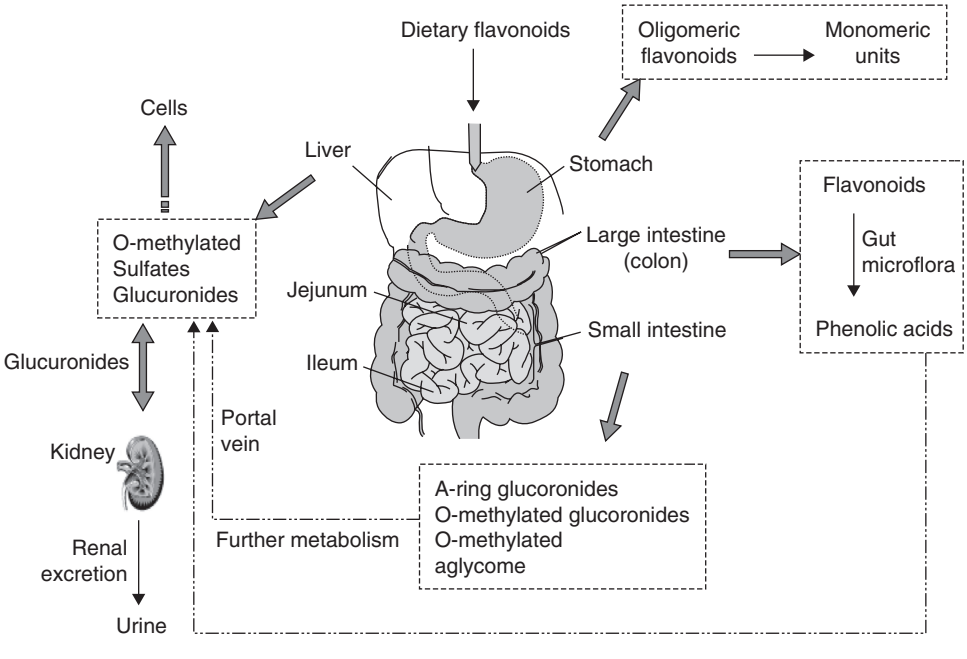

Figure 5.11. A diagrammatic representation of the various metabolic routes that determine flavonoid bioavailability after its ingestion through food.

quercetin glycosides were detected, and the plasma levels of the detected polyphenols ranged from 0.5 to 1.6 μM.

Only recently has it been proven that flavonoids from dietary sources are absorbed at an extent that may exert an antioxidant effect *in vivo* (Tsuda et al., 2000; Ramirez-Tortosa et al., 2001). The ability of flavonoids to act as H-donating compounds was believed to underlie many of their reported health effects. Yet the extent of the antioxidant potential *in vivo* is dependent on the absorption, metabolism, distribution, and excretion of these compounds within the body after ingestion and the reducing properties of the resultant metabolites. The GIT plays a very important role in the metabolism and conjugation of polyphenols. In the jejunum and ileum of the small intestine, nearly all flavonoids undergo glucuronidation to differing extents by uridine diphosphate (UDP)-glucuronosyl transferase enzymes. If the flavonoid has a catechol-containing B ring, there is also extensive O-methylation by the action of catechol-*O*-methyl transferase. Studies have shown that the extent of absorption of dietary polyphenols in the small intestine is relatively small (Spencer et al., 1999; Kuhnle et al., 2000). Unabsorbed flavonoids and those absorbed, metabolized in the small intestine and liver, and transported back into the intestinal lumen, reach the large intestine, where they are further metabolized by gut microflora to smaller phenolic acids. Bacterial enzymes may catalyze several reactions like hydrolysis, dehydroxylation, demethylation, ring cleavage, and decarboxylation. The extent to which phenolic acids are absorbed in the colon is unknown. However, they are detected in the plasma and are often further conjugated and metabolized in the liver. The rest of the compounds derived from intake of flavonoids are excreted through feces. Therefore, the major flavonoid conjugates and metabolites are O-glucoronidated, O-sulfated and, in the case of catechol structures, O-methylated derivatives (Spencer and Rice-Evans, 2003). The action of these flavonoid metabolites, in particular the O-methylated flavonoids, is of great interest. For instance, 3′-*O*-methyl epicatechin and epicatechin glucoronides protect against apoptotic cell death induced by H_2O_2 or oxidized LDL (Spencer et al., 2001; Schroeter et al., 2001). It has been reported that the metabolic conversion of flavonoids do not decrease the antioxidative ability of rat plasma, indicating that conjugated metabolites might participate in the antioxidant defense (Terao, 1999).

In terms of individual flavonoids, quercetin, probably due to its commercial availability, has been extensively studied. Murota and Terao (2005) reported that in the gastrointestinal mucosa of rats, quercetin, after being methylated and/or conjugated with glucuronide or sulfate, was transported in the lymph (not as aglycone). After extensive studies on rat and pig models, de Boer et al. (2005) reported that after 11 weeks of feeding quercetin, its metabolites were identified in many rat tissues, with the highest concentration found in lungs and the lowest in brain, white fat, and spleen. Several studies have reported the presence of various flavonoids in brain tissue of rats: naringenin, tangeretin, genistein, epicatechin, and anthocyanins (Andres-Lacuevea et al., 2005; Talavera et al., 2005). Anthocyanins (cyanidin-3-galactoside, cyanidin-3-arabinoside, malvidin-3-glucoside, malvidin-3-galactoside, malvidin-3-arabinoside peonidin-3-arabinoside, delphinidin-3-galactoside) were found in the cerebellum, cortex, hippocampus, and stratum of rats fed with blueberries for 10 weeks. This indicates that flavonoids can cross the blood–brain barrier and localize in various brain regions important for learning and memory (Andres-Lacuevea et al., 2005).

Paganga and Rice-Evans (1997) reported the absorption of flavonoids and their presence in human plasma in the glycosylated form. Rutin and other quercetin glycosides were detected, and the plasma levels of the detected polyphenols ranged from 0.5 to 1.6 μM.

Spencer et al. (1999) studied the perfusion of the jejunum and ileum in an isolated rat intestine model with flavonoids and hydroxycinnamates and the influence of glycosylation on the subsequent metabolism. It was reported that flavone and flavonol glucosides and their corresponding aglycones were glucuronidated during transfer across the rat jejunum and ileum, and this glucuronidation occurred without the need for gut microflora. Furthermore, the presence of glycosidases as well as UDP-glucuronyl transferase in the jejunum was suggested. In contrast, quercetin-3-glucoside and rutin were mainly absorbed unmetabolized. The results suggest that the highly reducing phenolics are absorbed predominantly as glucuronides, whereas monophenolic hydroxycinnamates and flavonoids with a monophenolic B-ring were less predisposed to glucuronidation. Hydroxycinnamates, which are esterified naturally (Macheix et al., 1990), are not cleaved in the gastric lumen (Rechner et al., 2001) nor the small intestine (Plumb et al., 1999), but in the colon by esterase activity of the gut microflora (Scheline, 1991). Subsequent absorption of the free hydroxycinnamic acids, ρ-coumaric, caffeic, and ferulic acid, has been reported (Jacobson et al., 1983) as well as O-methylation of caffeic acid to ferulic or isoferulic acid, and conjugation to glucuronides or sulfates (Bourne and Rice-Evans, 1998). The bioavailability and metabolism of individual flavan-3-ols has been studied in animals and humans demonstrating the absorption and elimination of low micromolar amounts of their direct conjugate (Lee et al., 1995; Nakagawa and Miyazawa, 1997; van het Hof et al., 1999; Warden et al., 2001). The high methylation rate of catechin (95%) in the liver indicated intensive catechol-*O*-methyl transferase activity (Manach et al., 1999).

Due to their instability at higher pH values, the analysis of anthocyanins in body fluids is difficult, but absorption and excretion of very low proportions of the intact glycosides has been reported after ingestion of anthocyanin-rich berry or wine extracts (Lapidot et al., 1998; Miyazawa et al., 1999; Tsuda et al., 1999; Murkovic et al., 2000; Cao et al., 2001). In a study involving five healthy volunteers, 200 g blackberries (960 μmol of anthocyanin) were fed, and urine samples were analyzed. In addition to native cyanidin-3-glucoside, several other anthocyanin metabolites were identified in the urine: methylated glycosides, glucuronides of anthocyanidins and anthocyanins, a sulfoconjugate of cyanidin, and anthocyanidins (Felgines et al., 2005). Wu et al. (2002) studied the absorption and metabolism of anthocyanins in four elderly women fed with 12 g elderberry extract (720 mg total anthocyanin) and six elderly women fed 189 g lowbush blueberry (690 mg total anthocyanin). Two major anthocyanins in elderberry extract, viz., cyanidin-3-glucoside and cyanidin-3-sambubioside, together with four metabolites, peonidin-3-glucoside, peonidin-3-sambubioside, peonidin-monoglucuronide, and cyanidin-3-glucoside monoglucuronide were identified in urine within 4 hours of ingestion. The average elderberry anthocyanin excretion was 554 μg (0.08% intake in 4 hours). Urine samples of women fed with blueberry retained anthocyanins in the original form, and the total urinary excretion during the first 6 hours was 23 μg. Plasma anthocyanin levels were below detection limit, demonstrating low absorption of anthocyanins compared to other flavonoids. In addition, this study reports *in vivo* methylation of cyanidin to peonidin and the presence of glucuronide conjugates of anthocyanins. Talavera et al. (2005) measured anthocyanin distribution in the stomach, jejunum, liver, and kidney, as well as the brain in rats fed a blackberry anthocyanin-enriched diet for 15 days. The stomach tissue contained only native blackberry anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-pentoside), while in jejunum, liver, and kidney, native and methylated anthocyanins as well as conjugated anthocyanidins (cyanidin and peonidin monoglucuronides) were identified. Proportions of anthocyanin derivatives differed according to the organ considered, with the liver having the highest proportion of

methylated forms. This study demonstrated the presence of the aglycone form in jejunum and plasma. In the brain, total anthocyanin content (blackberry anthocyanins and peonidin 3-*O*-glucoside) reached 0.25 nmol/g of tissue. Additional studies on the concentration of flavonoids and their metabolites are needed to identify target tissues of the various flavonoids, which may help understand their mechanisms of action *in vivo* (Del Rio et al., 2010; Williamson and Clifford, 2010).

5.12 DIETARY POLYPHENOLS AND PREVENTION OF DISEASES

Polyphenols are common constituents of foods of plant origin and are major antioxidants of our diet (Middleton and Kandaswami, 1993; Scalbert et al., 2005). Fruits such as apple, grape, pear, cherry, and various berries contain up to 200–300 mg polyphenols per 100 g fresh weight. A glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. The total dietary intake is estimated at about 1 g/d, but may vary considerably based on the dietary patterns. This level is much higher than the intake of all other known dietary antioxidants like vitamin C, vitamin E, and carotenoids. The two main types of polyphenols are flavonoids and phenolic acids. Some of the most common flavonoids are quercetin, a flavonol abundant in onion, tea, and apple; catechin, a flavanol found in tea and several fruits; hesperetin, a flavanone present in citrus fruits; cyanidin, an anthocyanin giving its color to many red fruits (blackcurrant, raspberry, strawberry, etc.); daidzein, the main isoflavone in soybean; proanthocyanidins, common in many fruits, such as apple, grape, or cocoa and are responsible for their characteristic astringency or bitterness. One of the most common phenolic acids is caffeic acid, present in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is the major phenolic compound in coffee. Polyphenols are proven antioxidants and may protect cell constituents against oxidative damage (Spencer et al., 2001). Research on the effects of dietary polyphenols strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers. The antioxidant properties of polyphenols have been widely studied, but it has become clear that the mechanisms of action of polyphenols go beyond the modulation of oxidative stress.

5.12.1 Polyphenols and cardiovascular diseases

Cardiovascular disease is the leading cause of death in the United States, Europe, and Japan (Moreno and Mitjavila, 2003). Atherosclerosis is a disease of the arteries involving a local thickening of the vessel wall caused by three types of pathological entities: foam cells, fatty streaks, and fibrous plaques. A stroke or myocardial infarction occurs when the lumen of the vessel becomes completely occluded by a thrombus formed at the site of a plaque. The following mechanisms have been observed: (1) increased recruitment of circulating monocytes into the vessel intima by the chemoattractant force of oxidized LDL (Frostegard et al., 1991); (2) enhanced rates of oxidized LDL uptake and degradation by the macrophage through the receptor (Henriksen et al., 1981); (3) the oxidation of the LDL particle inhibits macrophage exodus from the artery (Quinn et al., 1987); and (4) cellular injury caused by oxidized LDL (Cathcart et al., 1985). These observations have led to the assumption that *in vivo* LDL may be oxidized by free radicals, leading to the atherosclerotic process. Elevated amounts of oxidized LDL are present in the blood of patients with

atherosclerotic disease (Holvoet et al., 1995). A number of animal studies have demonstrated that the consumption of polyphenols limits the development of atheromatous lesions. Supplementation of drinking water with dealcoholized wine, pomegranate juice, catechins, or quercetin reduced the size of these lesions in apoE-deficient mice (Hayek et al., 1997; da Silva et al., 1998; Liao and Yin, 2000; Kaplan et al., 2001; Miura et al., 2001). These effects are associated with reduced low LDL uptake by macrophages, lower oxidation of isolated LDL, and decreased susceptibility of LDL to aggregation. Similar results were obtained through supplementation of a cholesterol-enriched diet with an extract of grape seeds rich in proanthocyanidins and administered to rabbits (Yamakoshi et al., 1999). In man, the lower levels of oxidation products of phosphatidylcholine (the main lipid found in LDL) observed after consumption of green tea catechins suggest that polyphenols effectively protect LDL against oxidation (Nakagawa et al., 1999). Polyphenols may exert antithrombotic effects. They inhibit platelet aggregation *in vitro* (Sagesaka-Mitane et al., 1990; Russo et al., 2001). They were also shown to inhibit platelet aggregation in several animal models: the consumption of polyphenol-rich red wine in the rat prevented the platelet rebound effect (Ruf et al., 1995). The consumption of red wine or nonalcoholic red wine reduces bleeding time and platelet aggregation induced by collagen in the rat (Wollny et al., 1999).

Polyphenols can improve endothelial dysfunction, an early event in atherogenesis. The endothelium-dependent vaso-relaxing activity of isolated polyphenols, such as wine anthocyanins (Andriambeloson et al., 1998), soy isoflavones (Honor′e et al., 1997), resveratrol, quercetin (Chen and Paceasciak, 1996), and cocoa proanthocyanidins (Karim et al., 2000) has been observed on isolated rat or rabbit aorta. The consumption of black tea (450 mL) increased artery dilation 2 hours after intake by coronary patients (Duffy et al., 2001). A similar improvement of endothelial function was observed when the same patients consumed 900 mL of tea/day for 4 weeks. The consumption of 240 mL red wine during 30 days also counteracted the endothelial dysfunction induced by a high-fat diet (Cuevas et al., 2000). Both the consumption of tea and a moderate consumption of wine have been regularly associated to a lower risk of myocardial infarction in both case-control and cohort studies (Peters et al., 2001; Rotondo et al., 2001).

5.12.2 Polyphenols and cancer

Fruit and vegetable consumption has been associated with reduced risk of cancer development (Steinmetz and Potter, 1996; Cohen et al., 2003). Different mechanisms have been suggested to explain the anticarcinogenic effects of polyphenols. First, polyphenols may act as blocking agents at the initiation stage by influencing the metabolism of procarcinogens and modulating the expression of cytochrome P450 enzymes involved in their activation to carcinogens. They may also facilitate their excretion by increasing the expression of phase II conjugating enzymes. The absorbed polyphenols could then activate these enzymes for their own detoxification and thus, induce a general boosting of our defenses against toxic xenobiotics. Polyphenols may also limit the formation of initiated cells by stimulating DNA repair. Second, polyphenols can act as suppressing agents, and inhibit the formation and growth of tumors from initiated cells; they inhibit cell proliferation *in vitro* (Agullo et al., 1996; Kuntz et al., 1999). It was also shown that some polyphenols can affect growth-related signal transduction pathways through inhibition of protein kinase C and AP-1-dependent transcriptional activity (Dong et al., 1997). They inhibit oncogene expression and the activity of ornithine decarboxylase, a key enzyme in the synthesis of

polyamines associated with cell proliferation. Phenolic phytoestrogens could influence the growth of hormone-responsive tumors through their antiestrogenic properties or their capacity to affect the response to endogenous estrogens (Kuiper et al., 1998). This may explain the protective effects of isoflavones against mammary and prostate cancers observed in different animal models (Lamartiniere et al., 2002). Polyphenols can induce apoptosis of tumor cells and, therefore, reduce the growth of tumors (Clifford et al., 1996; Damianaki et al., 2000).

Various antioxidants, including polyphenols, inhibit nuclear factor kappa-B (NF-kB) activation, probably through triggering a redox-sensitive signal in the cells (Flohe et al., 1997). The inhibition of such transcription factors by polyphenols may play an essential role in the prevention of cancers. Polyphenol supplements might be useful as adjuvants in chemotherapy or radiotherapy treatments. Some polyphenols were shown to reinforce the antiproliferative activities of anticancer drugs. The (-)-epigallocatechin-3-gallate (EGCG) showed synergistic effects with sulindac or tamoxifen on apoptosis of the lung cancer cell line PC-9 (Suganuma et al., 1999), and quercetin potentiated the growth inhibition of ovarian cancer cells and leukemia cells by cisplatin (Scambia et al., 1990; Cipak et al., 2003). Clinical trials will be needed to establish adjuvant effects of the most promising polyphenols in cancer patients.

5.13 INCREASING HEALTH BENEFICIAL PROPERTIES OF JUICES

A major drawback of processed fruit/vegetable preparations is their low phospholipid content. During fruit ripening, phospholipase D and associated enzymes that catabolize the membrane phospholipids are activated, thereby generating neutral lipids (Paliyath et al., 2008). Essentially, this is a common feature of the ripening process and hence, there is a reduction in the available phospholipids in any food preparations derived from fruits or vegetables. Currently, there are no procedures adopted to enhance the phospholipid levels of processed fruit/vegetable preparations. Since there is an increase in the demand for fruit/vegetable juice preparations, it will be advantageous if the phospholipid content could be enhanced that would in turn enhance product quality and healthfulness of the processed product. As well, during the preparation of clarified juice, there is a large degree of waste (skin, fiber, etc.) generated.

Tomatoes are the fourth most commonly consumed fresh fruit and the most frequently consumed canned fruit in the American diet (Canene-Adams et al., 2004). According to the World Processing Tomato Council's (WPTC) 2007 report, the United States produced 10,950,000 MT and Canada 563,000 MT of tomatoes that are used for processing (www.wptc.to/Releases-wptc.aspx). More than 80% of processed tomatoes are consumed in the form of tomato juice, paste, puree, sauce, and salsa. The consumption of tomato has increased over the past three decades because of an increased knowledge of the nutritional and processing qualities of tomato and tomato products. Tomatoes contain many bioactive components such as vitamins C and E and carotenoids. Lycopene, the main carotenoid in tomatoes, is believed to be responsible for the positive health effects associated with increased tomato intake. The characteristic deep-red color of ripe tomato fruits and tomato-based foods is mainly due to lycopene. There is emerging epidemiology data supporting the connection between increased tomato consumption and reduced risk for both cardiovascular disease and prostate cancer (Giovannucci, 1999; Petr and Erdman, 2005).

Despite wide application of phospholipids in the food industry as emulsifiers, their application in processing of fruits and vegetables is limited. Lecithin is a naturally occurring mixture of phosphatidyl choline with diverse fatty acid side chains such as stearic, oleic, palmitic acids. It is a significant constituent of nerve tissue and brain and is the predominant phospholipid in most mammalian membranes. Commercial sources of lecithin include vegetable oilseeds such as soybeans, rapeseeds and sunflower seeds, and animal sources such as egg yolk, milk, and brain tissue. The phospholipid content of plant raw materials is usually in the range of 0.3–2.5% (on dry basis) while animal sources have substantially higher levels ranging from 2 to 14% (Schneider and Virmani, 2001). Lecithin is widely used in the food industry as an emulsifier, lubricant, viscosity reducer, and as an anti-spattering, wetting, and release agent (Rossi, 2007). Relatively small amounts of lecithin are needed in foods (0.1–2%) and at these low levels of usage, the color, odor, and flavor of lecithin are not normally noticeable. It has "generally recognized as safe," (GRAS) status in foods with no limitation other than current good manufacturing process (Schneider and Virmani, 2001).

Phosphatidylcholine (PC), the most nutritionally significant phospholipid in lecithin, is involved in a myriad of essential metabolic reactions. Some of these include (1) providing structural stability to cell membranes; (2) providing a reserve supply of choline and acting as a second messenger; (3) constituting membranes of lipoproteins; and (4) synthesis of acetyl choline. Dietary Reference Intake (DRI) for PC is 425 mg/day (females) and 550 mg/day (males) (National Academy of Sciences Food and Nutrition Board, 1998). PC protects liver from damage associated with long-term alcohol consumption such as fatty liver (Lieber et al., 1990), while a choline (PC)-deficient diet enhances the development of abnormalities, including liver carcinogenesis (Copeland and Salmon, 1946; Salmon et al., 1955; Goshal and Farber, 1984; Rogers, 1995). Lecithin lowers cholesterol by decreasing the absorption of dietary cholesterol from the intestine to the bloodstream (Beil and Grundy, 1980; Rampone and Machida, 1981), and by increasing the synthesis of bile salts, thereby reducing the cholesterol available to reach the bloodstream that can cause damage to the blood vessels (Mastellone, 2000). In a developing fetus, choline plays a critical role in the development of brain areas such as hippocampus and septum, which are thought to be heavily involved in the formation and retrieval of memories (Albright, et al., 1999a,b). Studies have shown that choline ingested in the form of lecithin, or its precursor cytidine 5′diphosphocholine (CDP-choline), improved both immediate and logical memory in addition to improving mild memory loss associated with the aging process (Spiers et al., 1996; Alvarez et al., 1997).

Recent experiments have shown that it is possible to enrich fruit juices with the addition of soy lecithin (Oke et al., 2010). Soy lecithin can be added during processing of tomato and grape juice at concentrations up to 1%. In tomato juice preparations containing soy lecithin, the quality characteristics such as viscosity and stability of lycopene were achieved. In juices such as tomato juice that are enriched in pectin, the addition of soy lecithin will provide additional food components such as proteins and lipids that can result in stable food matrix interactions. The effect of soy lecithin was not as pronounced in grape juice. The presence of lipid components in tomato can stabilize lycopene during processing and may enhance its bioavailability, as lycopene has been found to be absorbed preferentially from an oil base. Using an *in vitro* digestion model, a high degree of stability was observed for carotenoids and tocopherols that exceeded 70%, and were available for the formation of absorbable micelles (Granado-Lorencio et al., 2007). Carotenoid esters such as xanthophyll esters were resistant to pancreatic lipase, but were de-esterified

by cholesterol esterase. Cholesterol esterase appears to be an enzyme capable of releasing a wide variety of bioactive components from their natural esters (40–60%). Complex formation with the food matrix reduced the accessibility of the enzyme to the components resulting in a lower degree of release. Juices and extracts allowed a higher degree of enzyme accessibility and better hydrolysis of the ester forms. Carotenoid absorption from foods is highly favored from micelles, and components such as phospholipids and other lipids, bile salts, and physicochemical conditions in the digested food influence micelle formation. Thus, addition of components such as phospholipids that enrich micelle formation in juices appears to be beneficial for the bioavailability of hydrophobic nutraceutical components.

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6 Cruciferous Vegetable-Derived Isothiocyanates and Cancer Prevention

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6.1 INTRODUCTION

Human malignancies are the second leading cause of deaths worldwide, affecting all age groups and genders. Chemoprevention is a cancer preventive strategy to suppress, delay, or reverse the onset of carcinogenesis. So far, the major treatment modalities such as surgery, chemotherapy, or radiotherapy used to treat human malignancies are associated with side effects. Thus, chemoprevention of human cancers using naturally occurring dietary components has been of great interest recently. The advantage of using a bioactive dietary agent is that it is cost-effective and is usually associated with no side effects. Isothiocyanates (ITCs) belong to a group of organosulfur compounds present in all the brassica (cruciferous) vegetables such as broccoli, cauliflower, and cabbage. Accumulating epidemiological and case-control studies suggest that consumption of cruciferous vegetables reduces the risk of developing human cancers. Cruciferous vegetables are a rich source of glucosinolates, which are converted into isothiocyanates (ITCs) by the enzyme myrosinase or by intestinal flora in the body. ITCs exert anticarcinogenic effects mainly through the inhibition of phase I carcinogen-metabolizing enzymes and/or induction of phase II detoxifying and cellular-defensive enzymes. Several recent studies have demonstrated that these naturally occurring ITCs inhibit the growth of malignant cells and *in vivo* tumorigenesis by targeting multiple signaling pathways leading to increased apoptosis. The differential effects of ITCs on apoptosis induction have been found to be associated with the molecular structures of different ITCs and the nature of carcinogens involved. This review addresses the biological impact of various ITCs on the induction of cell death/apoptosis mediated through multiple signaling pathways.

Human malignancies are one of the leading causes of deaths worldwide, with the United States being no exception (Polednak, 1994). The mortality due to human malignancies has been correlated with the difficulties in detection and prognosis of cancers at an early stage (National Academy of Sciences, 1982). At a later stage, treatment becomes

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almost impossible because the malignant cells frequently become resistant to currently available treatment modalities, including chemotherapy and radiotherapy (National Academy of Sciences, 1982). Several case-control and molecular studies have indicated that the occurrence and incidence of human malignancies are prevalent and prominently associated with multiple genetic and epigenetic abnormalities, including alterations in DNA sequences, changes in chromosomal copy numbers, and hypermethylation. These events result in the activation of oncogenes and inactivation of tumor-suppressor genes (Keum et al., 2004; Lampe, 2007; Nakamura, 2009). Chemoprevention of human malignancies is a new and promising strategy to prevent, inhibit, block, retard, or reverse the process of carcinogenesis using naturally occurring agents. The chemopreventive properties of naturally occurring dietary compounds are due to their antioxidative and anti-inflammatory activities. Further, dietary phytochemicals block the promotion phase of carcinogenesis process by causing cell cycle arrest and apoptosis in the malignant cells through multiple signaling pathways (Keum et al., 2004; Zhang et al., 2005; Wu and Hua, 2007). Among various dietary components, cruciferous vegetables which are rich in ITCs have been shown to be protective against human cancers (Tookey et al., 1980; Fenwick et al., 1989; Block et al., 1992; Fahey et al., 2001). Over several decades, a number of epidemiological, case-control, pharmacological, and molecular studies conducted in United States, Europe, Shanghai, Singapore, the Netherlands, and Canada have indicated an inverse relationship between the intake of cruciferous vegetables and the risk of human malignancies, including those of the head and neck, lung, breast, liver, pancreas, gastrointestinal tract, colon, prostate, ovary, and blood (Bueno de Mesquita et al., 1991; Olsen et al., 1991; Block et al., 1992; Ji et al., 1995; Ketterer, 1998; Lin et al., 1998; Jain et al., 1999; Michaud et al., 1999; Cohen et al., 2000; Gamet-Payrastre et al., 2000; Kelloff et al., 2000; Kolonel et al., 2000; London et al., 2000; Spitz et al., 2000; Zhang et al., 2000; Bonnesen et al., 2001; Levi et al., 2001; Zhao et al., 2001; Seow et al., 2002; Fowke et al., 2003a; Ambrosone et al., 2004; Zhang, 2004; Brennan et al., 2005; Kalkunte et al., 2006; Higdon et al., 2007). These dietary bioactive compounds have immense anticarcinogenic potential as they are not only part of the routine human diet but also have no side effects. ITCs are among the most active agents present in cruciferous vegetables such as broccoli, cabbage, turnip, cauliflower, kale, and brussels sprouts. ITCs mainly exist in a precursor form known as glucosinolate (β-thioglucoside *N*-hydroxysulfates), which is released by the action of the enzyme myrosinase (thioglucoside glucohydrolase) when the plant cells are damaged either by cutting or chewing (Tookey et al., 1980; Fenwick et al., 1989; Fahey et al., 2001; Hayes et al., 2008). ITCs are a group of compounds that exert their chemopreventive effects by inhibiting the activation of carcinogens through inhibition of phase I enzymes and/or induction of phase II enzymes involved in the process of detoxification (Zhang and Talalay, 1994; Hecht, 1996; Maheo et al., 1997; Hecht, 1999; Dick and Kensler, 2002). Various studies have suggested that ITCs are protective against chemically induced cancers in experimental animals through similar mechanisms (Chung, 1992; Zhang et al., 1992; Zhang and Talalay, 1994; Stoner and Morse, 1997; Hecht, 1999; Rushmore and Kong, 2002). It has been reported that the balance between phase I and phase II enzymes plays an important role in determining an individual's risk for cancer (Wilkinson and Clapper, 1997; Rushmore and Kong, 2002). Among different ITCs, some of the extensively studied ITCs are allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), and sulforaphane (SFN).

6.2 METABOLISM OF XENOBIOTICS

Most of the xenobiotics are subjected to metabolism. These enzymatic reactions mainly occur through oxidation and then by reduction and hydrolysis increase the hydrophilicity of substrates. The whole process is termed as phase I metabolism. This system offers protection not only against carcinogens present in the environment but also against drugs. The cytochrome P450 (CYP) constitutes a family of phase I enzymes which converts procarcinogens to highly reactive electrophilic carcinogen that have the potential to disrupt the genomic stability and damage DNA bases. So far, 57 CYPs have been identified in humans, based on similarities in genetic sequences and protein functions (Nelson et al., 1993). Phase I reaction occurs when ligands bind to aryl hydrocarbon (AH) receptor and the complex translocate to the nucleus to bind to the xenobiotic responsive elements (XREs) upstream of CYP genes (Hankinson, 1995). Jiao et al. (1996) have shown that cytochrome P450E1, *N*-dimethylnitrosoamine demethylase (NDMAd), and various isozymes play major roles in activating tobacco-specific nitrosamines and demonstrated that ITC conjugates could be used as potential chemopreventive agents (Jiao et al., 1996). It has been suggested that ITCs react with free thiols or glutathione (GSH) and form GSH conjugates, which have inhibitory effects toward rat and human liver microsomal NDMAd activity and cytochrome P450E1 (Kelsey and Bernstein, 1996; Conaway et al., 2005). Moreno et al. showed that BITC-mediated inactivation of cytochrome P450 2E1 was NADPH dependent and resulted in 65% loss of enzyme activity (Moreno et al., 1999). In another study, Steinkellner et al. reported that the inhibition of cytochrome P450 1A2 in human hepatoma cells HepG2 by ITCs protect against the genotoxic effects of benzo[α]pyrene (B[α]P) (Steinkellner et al., 2001). Among several CYP enzymes, cytochrome P4503A4 (CYP3A4) has been shown to be responsible for the increased activation of various hepatic and intestinal protoxicants, pharmaceutical compounds, and endogenous sterols, and its expression is mediated via nuclear hormone receptors (steroid and xenobiotic receptor; SXR, also named as hPXR). It plays an important role as a xenobiotic sensor to coordinately regulate xenobiotic metabolism through transcriptional regulation of xenobiotic detoxifying enzymes and its transporters. Zhou et al. reported that SFN is a specific antagonist of human SXR and inhibits SXR-mediated induction of drug clearance through the inhibition of SXR coactivator recruitment which represses SXR activities. SFN also inhibits SXR-mediated CYP3A4 expression and its catalyzed midazolam clearance in human primary hepatocytes (Zhou et al., 2007).

Phase II enzymes convert carcinogens into inactive metabolites that are readily excreted from the body and hence prevent further reaction/conjugation with DNA. This is an important process in chemoprevention. ITCs are well-known inducers of human phase II detoxification enzymes (Talalay et al., 1995; Brooks et al., 2001; Steinkellner et al., 2001), including glutathione *S*-transferases (GSTs), nicotinamide adenine dinucleotide phosphate (NADPH)-quinone oxidoreductase, and UDP-glucuronyltransferases (Zhang and Talalay, 1994; Meyer et al., 1995; Talalay et al., 1995; Hecht, 2000; Talalay and Fahey, 2001; Dinkova-Kostova et al., 2007). Among these, GSTs play a crucial role in detoxifying a wide variety of electrophilic xenobiotics and carcinogens primarily by catalyzing their conjugation with GSH, thereby facilitating their metabolism and excretion (Jana and Mandlekar, 2009). The cytosolic GSTs are constituted from at least 17 different subunits within seven classes: alpha (GSTA1-A5), mu (GSTM1-M5), omega (GSTO1, O2), pi (GSTP1), theta (GSTT1, T2), sigma (GSTS1), and zeta (GSTZ1). The protective effect

of cruciferous vegetables is likely to be influenced by GSTM1 genotype. Approximately 50% of the population regardless of their race has a deletion of GSTM1 gene. Epidemiological studies have indicated that those cancer patients who have one GSTM1 wild-type allele and consume broccoli gain greater protection than those cancer patients who have GSTM1 null alleles (Lin et al., 1998; Spitz et al., 2000; Joseph et al., 2004). Studies have indicated that phase II enzymes are transcriptionally induced by low concentrations of a wide variety of chemical agents, and this induction is important for the blockage of chemical carcinogens (Yu et al., 1999; Kong et al., 2001; Keum et al., 2006). The antioxidant response element or electrophile response element (ARE/EpRE) found at the 5′ flanking region of these phase II genes play an important role by mediating their induction in response to xenobiotics. Among the transcription factors, members of the basic leucine zipper (bZIP) transcription factor, Nrf2, have been shown to heterodimerize with Maf G/K and bind to ARE for its transcriptional activation (Kong et al., 2001; Casagrande et al., 2002; Keum et al., 2006). Some of the phase II gene inducers including phenolic antioxidants and ITCs such as SFN are involved in transcriptional activation of ARE-mediated reporter genes which then activate mitogen-activated protein (MAP) kinase pathway (Yu et al., 1999; Kong et al., 2001; Keum et al., 2006). In addition to GST, thioredoxin system is another major player of phase II enzyme inducer. The thioredoxin system is composed of thioredoxin (Trx), NADPH, and thioredoxin reductase (TrxR), and is distributed widely in prokaryotes and eukaryotes. It plays an important role in maintaining the redox state of the cells based on thiol-dependent thiol-disulfide exchange reaction which is crucial to control cellular growth by protecting cells against oxidative stress through scavenging of reactive oxygen species (ROS). The Trx system functions in the synthesis of deoxyribonucleotides for DNA synthesis both at the time of replication and repair by ribonucleotide reductase. The human thioredoxin promoter region directs expression of thioredoxin gene under both housekeeping conditions and during oxidative stress. There are direct links between the Trx system and the protein glutathionylation events (Casagrande et al., 2002). Direct reduction of mitochondrial glutaredoxin 2 by TrxR also plays an important role as the redox sensor (Johansson et al., 2004; Enoksson et al., 2005). SFN regulates thioredoxin reductase/thioredoxin redox system in HepG2 and MCF-7 cells which act as inducers of thioredoxin reductase and its substrate as depicted by enhancement in the mRNA and protein expression (Terry et al., 2001). Other studies on the dietary bioactive isothiocyanates have now revealed that these dietary agents are monofunctional inducers that boost only the phase II enzymes (Prochaska and Talalay, 1988; Cuendet et al., 2006).

The effects of ITCs are based on the structure of ITCs such as AITC, BITC, PEITC, and SFN, and the nature of carcinogen involved. Numerous studies have identified multiple signaling mechanisms by which ITCs exert chemopreventive effects to inhibit the growth of cancer cells. The findings of multiple studies on ITCs and their chemopreventive properties against various human malignancies have been summarized below.

6.3 ITCS AND INHIBITION OF CANCER

6.3.1 Pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer-related deaths, affecting approximately 38,000 people per year in the United States (Jemal et al., 2005). Inspite of the current

treatment modalities, the mortality rate remained constant, leaving horizon for the development of new drugs (DiMagno et al., 1999). Studies from our laboratory demonstrated that BITC inhibits the proliferation of human pancreatic cancer cells by causing G2/M cell cycle arrest and inducing apoptosis mediated through increased DNA damage (Zhang et al., 2006a). Apoptosis in these cells was associated with the modulation of Bax and Bcl-2 levels and inhibition of nuclear factor kappa-B (NFκB) DNA binding activity, leaving a new direction for further investigation of the plausible mechanisms of BITC action in these cells (Srivastava and Singh, 2004). Recently we observed the BITC-mediated growth inhibition of pancreatic cancer cells (BxPC-3, AsPC-1, Capan-2, MiaPaCa-2, and Panc-1) was associated with the inhibition of signal transducer and activator of transcription (STAT-3) pathway (Sahu and Srivastava, 2009). Members of the STAT family of transcription factors have been identified to play a crucial role in the expression of genes that are involved in cell survival, proliferation, chemoresistance, and angiogenesis (Ihle, 1996; Darnell, 1997). We demonstrated that BITC-mediated inhibition of STAT-3 was due to decreased STAT-3 DNA binding and transcriptional activity as pretreatment of BxPC-3 cells with protein synthesis inhibitor cycloheximide almost completely reversed the deleterious effects of BITC and rescued the cells from apoptosis (Sahu and Srivastava, 2009), indicating that BITC specifically target STAT-3 pathway in pancreatic cancer cells to induce apoptosis (Sahu and Srivastava, 2009). We further observed that BITC treatment significantly reduced the growth of pancreatic tumor xenografts in athymic nude mice as compared to the control mice. Moreover, the decreased tumor burden *in vivo* by BITC treatment was correlated with the decreased STAT-3 phosphorylation as well as total STAT-3 protein levels and increased apoptosis as compared to the control mice, suggesting the role of STAT-3 in BITC induced apoptosis in pancreatic cancer (Sahu and Srivastava, 2009). Interestingly, BITC treatment minimally affected the survival of nearly normal immortalized human pancreatic ductal epithelial (HPDE-6) cells and failed to activate (phosphorylate) STAT-3; neither decreased the basal level of STAT-3 protein, DNA binding, and transcriptional activity (Sahu and Srivastava, 2009). These studies indicate the specificity of BITC toward cancer cells. In a separate study we found that BITC treatment caused the activation of mitogen-activated protein kinase (MAPK) that is known to play a critical role in the regulation of apoptosis (Sahu et al., 2009a). We observed that BITC treatment that resulted in the phosphorylation of all the MAPK family members (extracellular signal-regulated kinase [ERK], c-Jun N-terminal kinase [JNK], and P38) in a dose- and time-dependent manner caused cell cycle arrest and induced apoptosis. The phosphorylation of MAPK was found to be associated with the generation of ROS and depletion of GSH, which are known to maintain the redox balance and thus homeostasis of the cells (Ghezzi, 2005). We demonstrated that silencing the expression of MAPK by MAPK-specific ShRNA or pretreatment of pancreatic cancer Capan-2 cells with MAPK-specific inhibitors abrogated the effects of BITC and significantly protected the cells undergoing G2/M cell cycle arrest and apoptosis (Sahu et al., 2009a). Pretreatment of Capan-2 cells with antioxidant *N*-acetyl cysteine (NAC) blocked ROS production, inhibited the activation of MAPK, and protected the cells from G2/M cell cycle arrest and apoptosis, suggesting the involvement of ROS in BITC-mediated decreased survival of pancreatic cancer cells (Sahu et al., 2009b). The above-mentioned effects were not observed in normal pancreatic HPDE-6 cells, indicating that BITC specifically targets cancer cells (Sahu et al., 2009b). Recent reports showed that radiotherapy used to treat patients with pancreatic cancer is often associated with systemic toxicity and development of resistance (Casagrande et al., 2007). We demonstrated that pretreatment of pancreatic cancer cells with BITC sensitizes the pancreatic cancer cells and

increases the apoptosis-inducing effects of γ-irradiation at low doses (Sahu et al., 2009a). Kuroiwa et al. demonstrated that *N*-nitrosobis (2-oxopropyl) amine (BOP)-induced pancreatic cancinogenesis was blocked by BITC and SFN treatment in golden Syrian hamsters (Kuroiwa et al., 2006). Nishikawa et al. demonstrated that PEITC also blocked BOP-induced pancreatic tumorigenesis in hamsters (Nishikawa et al., 1996, 1999). In a recent report, Basu et al. observed that BITC inhibits the proliferation of pancreatic cancer cells by modulating the expression of death receptors and RasGAP/Rac1 (Basu and Haldar, 2009).

6.3.2 Brain cancer

The number of deaths from brain cancer is increasing tremendously, and it is estimated that 22,000 new cases are diagnosed every year in the United States (Bredel et al., 2009). Among several grades and types of brain cancer, glioblastoma is the most common, prevalent, and deadliest form of brain cancer (Bredel et al., 2009). The current treatment options for brain cancer are mass-reductive surgery, radiation, and chemotherapy. Although chemotherapy has been beneficial over the other two therapies, it eventually is associated with uncontrolled proliferation of glioblastoma cells (Stewart, 2002). The use of dietary ITCs as a promising anticarcinogenic agent against human cancers gained particular interest in the field of chemoprevention (Park and Pezzuto, 2002). In this regard, SFN has been shown to induce apoptosis in T98G and U87MG human glioblastoma cells which was mediated through an increase in intracellular calcium ion and upregulation of calcium-ion dependent protease calpain. Induction of calpain activated the cleavage of caspase 12 which in turn cause the release of cytochrome c and sequential activation of caspase 9 and caspase 3 leading to apoptosis (Karmakar et al., 2006). In another report, Jadhav et al. demonstrated that ITC iberin inhibited the proliferation and induced apoptosis in human glioblastoma cells mediated by the activation of caspase 9 and 3 (Jadhav et al., 2007). The findings from these studies provide the basis that ITCs could be used as promising agents for the prevention/treatment of brain cancer.

6.3.3 Prostate cancer

Prostate cancer remained the leading cause of cancer-related deaths among men in the United States. Inspite of various preclinical, clinical, and molecular interventions, the etiology of prostate cancer is unknown (Nelson et al., 2003), providing an opportunity for further investigation of novel chemotherapeutic drugs. Epidemiological studies have indicated that consumption of cruciferous vegetables may reduce the risk of human cancers, including prostate (Kolonel et al., 2000). Several reports have suggested that ITCs present in cruciferous vegetables inhibit the proliferation and induce apoptosis in human prostate cancer cells *in vitro* and *in vivo* via multiple cell signaling pathways without affecting the viability of normal prostate cells. A study by Xiao and Srivastava showed that AITC exerts antiproliferative activity against human prostrate cancer cells by causing G2/M cell cycle arrest and inducing apoptosis, with minimal effect on normal prostate epithelial cells (Xiao et al., 2003). Furthermore, Srivastava et al. demonstrated that AITC treatment significantly inhibits the growth of prostrate tumor xenograft in nude mice and that the growth inhibition was associated with increased apoptosis and reduced mitotic activity (Srivastava et al., 2003). PEITC-mediated cell death by mitogen-activated protein kinase (MAPK) activation in p53-independent manner has been reported in PC3 human prostrate cancer cells (Xiao

and Singh, 2002). On the other hand, in various other studies, multiple mechanisms of SFN mediated-cell death were reported. SFN causes accumulation of cells in G(0)/G(1) phase, and these effects were linked with the upregulation of cyclin-dependent kinase inhibitor p27 expression (Shan et al., 2006) or via attenuation of expression of phosphorylated and dephosphorylated androgen receptors with the inhibition of expression of cyclin D1 in androgen-dependent human prostate cancer LNCaP cells (Chiao et al., 2002). In recent reports, SFN-induced cell death was found to be mediated by inhibiting histone deacetylase enzyme activity and by decreasing the expression of key proteins such as estrogen receptor alpha, epidermal growth factor receptor (EGFR-1), and EGFR-2 in human PC-3 xenograft models (Myzak et al., 2007). Autophagy (type II cell death) is another mode of cell death and inspite of the identification of 30 autophagy-related genes in yeast, the exact molecular mechanism of how it differs from apoptosis (type I cell death) in terms of cell death is not fully understood (Suzuki et al., 2007), but it appears to be protective against apoptosis in some cellular model such as lymphoma (Amaravadi et al., 2007). Recently, Bommareddy et al. have shown that PEITC induced autophagic cell death in human prostate cancer cells PC3 and LNCaP through the modulation of autophagic protein Atg5. This induction of Atg5 was mediated through the suppression of Akt-mTOR signaling by PEITC treatment, although how Atg5 connects autophagic and apoptotic responses are not clear (Bommareddy et al., 2009). In another study, it has been reported that SFN induces autophagy in PC-3 and LNCaP human prostate cancer cells which was related with upregulation and processing the recruitment of autophagosomes to microtubule-associated protein 1 light chain 3 (LC3) and inhibition of release of cytochrome c and apoptosis, which represents a defense mechanism of cells against SFN-induced apoptosis (Herman-Antosiewicz et al., 2006). Inhibitors of apoptosis proteins (IAPs) have been shown to overexpress in response to drug treatment and make cancer cells to become resistant to apoptosis. Choi et al. demonstrated that SFN-mediated cell death was enhanced by downregulating IAP and inducing apoptosis inducing factor-1 (Apaf-1) in LNCaP human prostate cancer cell line (Choi et al., 2007). Other than SFN, PEITC was shown to induce apoptosis in PC3 human prostate cancer cells and retard the growth of PC3 xenografts in athymic nude mice. The inhibition of prostate tumor growth was correlated with the induction of proapoptotic proteins Bax and Bid and mediated by disruption of mitochondrial membrane potential and ROS generation (Xiao et al., 2006a). In another study, PEITC treatment has been shown to inhibit the proliferation of DU145 prostate cancer cells and induced G2/M cell cycle arrest via inhibiting IL6/JAK/STAT3 pathway (Gong et al., 2009). Pretreatment of DU145 cells with NAC reverse the process and protected the cells from PEITC-induced apoptosis, suggesting the involvement of ROS in PEITC-induced apoptosis in this model (Gong et al., 2009). In a separate report, PEITC treatment was shown to decrease the expression of alpha and beta tubulins, which are components of microtubules in prostate cancer cells in order to induce apoptosis (Yin et al., 2009). Wang et al. showed that PEITC treatment inhibits the expression of histone deacetylases (HDACs) and c-Myc, a known repressor of transcription, and induces the activation of p21 gene in order to induce apoptosis in androgen-dependent LNCaP prostate cancer cells (Wang et al., 2008). In a recent report, human volunteers were fed broccoli-rich diet and then the changes in the global gene expression patterns in the prostate gland were quantified before, during, and after 12 months of broccoli-rich diet consumption. These studies demonstrated that after 6 months, there was a significant difference between GSTM1 genotypes in those who were on broccoli-rich diet, which was found to be associated with the induction of TGFβ1 and epidermal growth factor (EGF) pathways, suggesting that consuming broccoli interacts with GSTM1 genotypes and results in the complex changes

in the signaling pathways associated with inflammation and carcinogenesis in the prostate (Traka et al., 2008). Yao et al. showed that SFN induces apoptosis in DU145 prostate cancer cells via inhibiting hypoxia inducible factor, which is mediated through the activation and ERK and JNK signaling pathway (Yao et al., 2008). In another report, Barve et al. demonstrated that PEITC suppress the growth of *in vitro* PC-3 cells and *in vivo* PC3 tumor xenografts in immunodeficient nude mice via inhibiting Akt signaling pathway (Barve et al., 2008). Similarly, PEITC treatment activates ERK and JNK which in turn phosphorylates antioxidant responsive element (ARE)-regulated transcription factor Nrf2 and induces the expression of stress-responsive gene heme oxygenase-1 (OH-1) to suppress the growth of PC3 prostate cancer cells (Xu et al., 2006a, b). These studies indicate that consumption of cruciferous vegetables containing ITCs could be used to delay the onset as well as progression of human prostate cancer cells.

6.3.4 Lung cancer

Lung cancer is the most commonly diagnosed cancer worldwide, with nonsmall cell lung cancer (NSCLC) accounting for about 80% of all diagnosed cases (Basu and Haldar, 2009). Almost 900,000 new cases in men and 300,000 in women are diagnosed every year worldwide (Jemal et al., 2009). Among four subtypes of human lung cancers, adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma, the adenocarcinoma of the lung is more prevalent and is currently the most common type of lung cancer in the United States (WHO, 2003). Carpenter et al. observed that dietary ITCs that mediated reduced risk of lung cancer in African Americans and Caucasians was associated with deletion of GSTM1 (Carpenter et al., 2009). Lam et al. investigated the association between cruciferous vegetable intake and the polymorphisms in GSTM1 and GSTT1 genes with an emphasis on lung cancer and found that it is weakly and inversely related with lung cancer risk. However, patients with homozygous deletion in GSTM1 and GSTT1 genes are strongly protected from lung cancer risk via cruciferous vegetable intake (Lam et al., 2009). Cigarette smoking has been one of the major risk factors increasing the likelihood of getting lung cancer in both the genders (Freedman et al., 2008). Epidemiological studies showed a 2.5-fold increased risk to the family members of a lung cancer patient after controlling for tobacco smoke, suggesting that genetic factors other than those related to metabolizing carcinogens from tobacco smoke may influence a person's susceptibility to lung cancer (Amos et al., 1999; Mitsuo et al., 2007). The compounds present in cigarette smoke involved in the induction of cancer risk are the complexes of polycyclic aromatic hydrocarbons typified by B[α]P and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Amos et al., 1999; Mitsuo et al., 2007). Chemoprevention using dietary compounds such as PEITC has been shown to reduce the risk of cigarette smoke–induced lung cancer in ex-smokers, passive smokers, and currently addicted smokers who fail to quit smoking (Brennan et al., 2005; Higdon et al., 2007; Sapone et al., 2007; Ye et al., 2007; Kim and Park, 2009). Balansky et al. demonstrated that when neonatal mice (strain H) were exposed to cigarette smoke for 120 days starting from birth and then fed with either PEITC or NAC or budesonide for another 90 days significantly protected the mice (male and female) from CS-induced pulmonary carcinogenesis (incidence of benign and malignant lung tumors) (Balansky et al., 2010). In another study, PEITC was shown to inhibit NNK-induced lung tumorigenesis in rodents by inhibiting activation of NNK (Hecht, 1997). Wattenberg et al. reported that BITC protects against lung cancer induced by B[α]P in A/J mice (Wattenberg, 1987). Lin et al. compared the chemopreventive

activities of BITC and PEITC on B[α]P-induced lung tumorigenesis in A/J mice. They administered BITC or PEITC by oral gavage 15 minutes before gavage of B[α]P and followed the same protocol three times at 2-week intervals and terminated the experiments after 26 weeks of first treatment. They observed that BITC but not PEITC substantially inhibited B[α]P-induced lung tumorigenesis, which was correlated with selective inhibition of CYP enzymes involved in the metabolic activation or detoxification of B[α]P (Lin et al., 1993). In a separate study, Yang et al. demonstrated that *N*-acetyl cysteine conjugate of phenethyl isothiocyanate (NAC-PEITC) caused increased apoptosis in A549 lung cancer cells predominantly by arresting the cells in G2/M phase of the cell cycle (Yang et al., 2005). Conaway et al. reached a similar conclusion that PEITC, SFN, and their NAC conjugates inhibited the progression of lung carcinoma induced by tobacco carcinogens in A/J mice (Conaway et al., 2005). Jin et al. found that SFN-induced apoptosis in A549 lung adenocarcinoma cells, when treated in combination with tumor necrosis factor-related apoptosis-inducing ligands (TRAILs), sensitizes TRAIL-resistant A549 cells to TRAIL-mediated apoptosis. This effect was due to chromatin condensation, DNA fragmentation and accumulation of apoptotic body in sub-G1 phase, downregulation of ERK and Akt leading to increase in caspase 3 activity (Zhang et al., 2003; Jin et al., 2007). These studies suggest that ITCs exhibit promising chemopreventive activity against lung cancer.

6.3.5 Breast cancer

Inspite of the availabilities of the modern screening and treatment options, the mortality rate due to breast cancer remained the leading cause of cancer-related deaths in women (Jemal et al., 2008). Among the risk factors, family history, early menarche, atypical hyperplasia of the breast, late age full-term pregnancy, late menopause, and Li–Fraumeni syndrome are the main factors (Kelsey and Bernstein, 1996). Among the current therapies used to treat malignancy of the breast, tamoxifen and estrogen receptor modulators, are commonly used. However, this cannot be given to the estrogen receptor negative breast cancer patients and is often associated with the adverse side effects, such as premenopausal symptoms and cataract (Cuzick et al., 2002), pointing toward an urgent need for the development of safe and targeted molecular therapies. Epidemiological and case control studies have indicated that consumption of cruciferous vegetable is inversely associated with the risk of breast cancer (Fowke et al., 2003b; Ambrosone et al., 2004; Zhang, 2004; Seow et al., 2005). Studies have shown that BITC effectively suppresses the growth of human breast cancer cells (MDA-MB-231, MCF-7) either by causing G2/M cell cycle arrest with a decrease in the levels of G2/M regulatory proteins or by apoptosis due to increased expression of pro-apoptotic proteins Bax and Bak and downregulation of anti-apoptotic protein Bcl-2 (Xiao et al., 2006b). Inhibition of mitochondrial respiratory complex III activity via ROS production (Xiao et al., 2008) and the growth of breast cancer tumors in MMTVneu mice by BITC have also been reported (Warin et al., 2009). On the contrary, normal mammary epithelial cell line (MCF-10A) were significantly resistant to growth arrest and apoptosis induction by BITC (Xiao et al., 2006b). Wattenberg et al. reported that BITC protects against mammary cancer induced by 7,12-dimethylbenz[a]anthracene (DMBA) in experimental animals.(Wattenberg, 1977). In recent reports, SFN-induced cell death was found to be mediated by the inhibition of the histone deacetylase enzyme activity and by decreasing the expression of key proteins such as estrogen receptor alpha, EGFR-1 and EGFR-2, in human breast cancer cells (Pledgie-Tracy et al., 2007). In a separate study, Rose et al. demonstrated that ITCs derived from broccoli and watercress significantly

inhibited the invasiveness of MDA-MB-231 breast cancer cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) via inhibiting matrix metalloproteinase-9 (MMP-9) activity (Rose et al., 2005).

6.3.6 Colon cancer

Colon cancer is the third leading cause of cancer-related death in the United States affecting both men and women, with 106,100 new cases diagnosed in 2009 (Horner et al., 1975–2006). The etiology of colon cancer is not fully understood. It could be multifactorial including genetic, environmental, and dietary origins. Among the risk factors, inflammatory bowel disease such as ulcerative colitis and Crohn's disease have been strongly associated with increased risk of colon cancer (Itzkowitz and Yio, 2004). The treatment strategies of colon cancer involve chemotherapy with cytotoxic agents such as irinotecan, 5-fluorouracil, and oxaliplatin, which often is associated with adverse side effects (Chau and Cunningham, 2002; Au et al., 2003; Gulec and Fong, 2007; Merlin et al., 2008; McConnell et al., 2009). The consumption of ITCs containing cruciferous vegetables has been shown to exhibit promising chemopreventive activity against human cancer, including colorectal cancer. In this regard, Kirlin et al. demonstrated that consumption of ITCs containing vegetables induced GSTs and related detoxifying enzymes through the induction of phase II enzymes in HT29 colon carcinoma cells mediated by enhanced elimination of chemical carcinogens (Kirlin et al., 1999). Studies conducted by Patten et al. revealed that BITC prevented DNA mutations and reduced the risk of cancer by activating the human quinone reductase (QR) gene which contains cis-acting AP1 and NF-κB transcription factor binding sites through the activation of c-Jun, a component of AP1 via the induction of JNK (Patten and DeLong, 1999). It has been reported that AITC exerts cytotoxic and cytostatic effects on HT29 human colon carcinoma cells and protects against the development of colorectal cancer by inhibiting progression of transformed cell clones selectively within the gastrointestinal mucosa (Musk and Johnson, 1993).

6.3.7 Hepatic cancer

The data from multiple studies suggest that the percentage of mortality due to hepatic cancer is rapidly increasing, affecting approximately 600,000 people annually, indicating the lack of proper diagnosis and treatment (McGlynn and London, 2005; Parkin, 2006; Wild and Montesano, 2009). Several preclinical, pharmacological, and molecular studies demonstrated that consumption of ITCs abundantly present in cruciferous vegetables is associated with reduced risk of hepatic cancer (Li et al., 1997; Laky et al., 2002; Scharf et al., 2003; Uhl et al., 2003; Hwang and Jeffery, 2005; Hwang and Lee, 2006). In this regard, Hwang et al. showed that AITC and *N*-acetlycysteine conjugate of AITC (NAC-AITC) suppressed the growth of SK-Hep 1 human hepatoma cells in a dose-dependent manner and downregulated the expression of MMPs and tissue inhibitors of metallopreteinases (TIMPs), which causes inhibition of cancer cell adhesion and migration (Hwang and Lee, 2006). In another report, the same group compared the chemopreventive activity of SFN and its NAC conjugate (SFN-NAC) on the growth inhibition and induction of phase II enzyme QR. It was found that both SFN and SFN-NAC caused dose-dependent growth inhibition and QR induction; however, the growth inhibition and QR induction by SFN-NAC was greater than SFN (Hwang and Jeffery, 2005). Recently, Hcharf et al. demonstrated that BITC treatment to human hepatoma HepG2 cell line increased GSH level,

which was mediated by the induction of γ-glutamylcysteine synthetase (GCS), the rate limiting enzyme of GSH synthesis, suggesting that this may contribute to chemoprevention against cancer (Scharf et al., 2003). Uhl et al. found that AITC, a breakdown product of sinigrin, which is the most abundant glucosinolate in mustard sprouts and brussel sprouts reduced the genotoxic effects of B[α]P, a carcinogen in a dose-dependent manner as measured by single cell gel electrophoresis (SCGE) in HepG2 cells and increased GSH levels in human hepatoma cells (Uhl et al., 2003). In a separate report, Laky et al. reached a similar conclusion and showed that AITC treatment reduced the genotoxic effects B[α]P-induced DNA damage in a dose-dependent manner which was mediated through the induction of activities of ethoxyresorufin *O*-deethylase (EROD) and GST at dose levels which were protective toward B[α]P-induced DNA damage in human HepG2 cells (Laky et al., 2002). Li et al. showed that PEITC treatment significantly decreased acetaminophen (ACAP)-induced hepatoxicity in Swiss-Webster mice. They found that PEITC treatment prevented the depletion of hepatic GST levels caused by conjugation of oxidized *N*-acetyl-para-amino-phenol (APAP) metabolites by inhibiting the rate of APAP-GSH formation as well as the P450 2E1-dependent *N*-nitrosodimethylamine demethylase and the P450 1A2-dependent ethoxyresorufin *O*-deethylase activities which are involved in the bioactivation of APAP (Li et al., 1997), suggesting that the consumption of ITCs is beneficial in reducing the risk of hepatic cancer.

6.3.8 Bladder cancer

Bladder cancer is one of the most expensive cancer to treat in the United States because it is often associated with extended courses of treatment coupled with the necessity for frequent surveillance examinations (Busby and Kamat, 2006). Studies have suggested that the individuals with GST or NAD(P)H: quinone oxidoreductase 1 (NQO1) gene variations that yield either a null or a suboptimal phenotype are at higher risk to bladder cancer compared to those with a wild-type genotypes (Salagovic et al., 1999; Park et al., 2003). In this regard, consumption of cruciferous vegetables has been shown to be inversely associated with a reduced risk of bladder cancer (Zhang et al., 2006b; Munday et al., 2008a; Tang et al., 2008). Zhang et al. demonstrated that consumption of broccoli sprouts induces both GST and NQO1 in cultured bladder cells *in vitro* and in rat bladder tissues *in vivo* which was mediated by the induction of Nrf2 transcription factor (Zhang et al., 2006a). Munday et al. compared the activities of 25 ITCs and demonstrated that consumption of those ITCs with 3-5-carbon aliphatic side chain with a methyl group attached to the alpha carbon particularly 1-methylbutyl ITC and AITC significantly lowered the incidence of bladder cancer by the induction of both the enzymatic activities (phases I and II) in *in vitro* and *in vivo* models (Munday and Munday, 2002, 2004; Munday et al., 2006, 2008b). Similarly, another report also documented the similar mechanisms of apoptosis induction in bladder cancer cells (Tang et al., 2006a). In another study, c-Jun activation by ITCs was implicated in the suppression of bladder cancer development mediated by AP-1 transcriptional activation (Li et al., 2005). On the contrary, another group reported that PEITC and its isoform (BITC) acts as strong promoters for genes involved in urinary bladder carcinogenesis. In this study, F344 male rats were first treated with diethylaminenitrosamine (DEN) and then after 2 days were administered with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) and thereafter placed on diet containing PEITC or BITC for 32 weeks. It was found that occurrence of papillary or nodular (PN) hyperplasia and carcinomas of urinary bladder were significantly elevated by PEITC or BITC after DEN and BBN

treatment. In another group of mice without DEN and BBN treatment, PN hyperplasia was present in all the rats treated with PEITC and BITC along with papillomas and carcinomas, and the growth of tumors and PN hyperplasias was reduced, suggesting that PEITC and BITC could be strong promoters of urinary bladder carcinogenesis with complete carcinogenic potential (Kolonel et al., 2000), raising the question whether ITCs behave differently in the bladder cells. In this regard, Tang et al. suggested that the carcinogenic potential of ITCs could be due to the prolonged exposure of the bladder epithelium to the high concentrations of electrophilic ITCs in the urine as *N*-acetylcysteine conjugates (NAC-ITCs) where the tumors originate. They further demonstrated that those ITCs that promoted tumor growth in rodent bladder induced apoptosis via arresting the cells in G2/M or S phase of the cell cycle in two human bladder cancer cells UM-UC-3 and T24 at low concentrations. However, these effects were abolished at higher concentrations, suggesting that the concentrations of urinary ITCs should be at low levels in order to prevent bladder cancer (Tang and Zhang, 2004). In another report, it was found that the consumption of broccoli extracts containing ITCs, particularly SFN, has been shown to induce apoptosis in bladder cancer cells by arresting the cells in S and M phases of the cell cycle, which was mediated through the mitochondrial death pathway (release of cytochrome c and sequential activation of caspase 9 and caspase 3) that in turn cleaved the inhibitors of caspase-activated-DNAse (ICAD) (Tang et al., 2006b). Shan et al. reported that SFN inhibits the proliferation of T24 bladder cancer cells by arresting them in G0/G1 phase of the cell cycle mediated through the upregulation of cyclin-dependent kinase inhibitor p27 expression (Shan et al., 2006). These studies suggest that ITCs could be used for the chemoprevention of bladder cancer.

6.3.9 Multiple myeloma (MM)

Blood cancer is a malignancy of B cells characterized by the latent accumulation of secretary and malignant plasma cells in the bone marraow (Hallek et al., 1998). It is one of the most invasive and deadliest forms among other human cancers. The National Institute of Health's Survillance Epidemiology and End Results (SEER) indicated that in 2006, 16,570 new cases of MM were diagnosed and 7320 MM-related deaths were reported in the United States. Despite several advances in the treatment modalities and the use of novel chemotherapeutic drugs, the outcome from this disease is very poor and is often associated with the relapsed/refractory diseases, indicating the need for the investigation of novel chemotherapeutic agents without any adverse side effects (Ries et al., 2008). Underlying studies indicate the fact that ITCs could be used for the intervention of MM. It is a well-known fact that induction of apoptosis is mediated by caspase 3 cleavage and subsequently by upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2 proteins. Multiple evidences exist for the overexpression of Bcl-2 oncoprotein-mediated resistance to apoptosis against a wide range of cytotoxic agents. Thomson et al. demonstrated that treatment with PEITC overcomes the inhibitory action of overexpressed Bcl-2 and triggers apoptosis in Jurkat cells (Thomson et al., 2006). Fimognari et al. confirmed that the extent of apoptosis mediated by SFN using thymidine block synchronized cells (T-leukemia cells) revealed that most of the cells which were sensitive to SFN were in the G phase and were less sensitive in the G2/M phase, suggesting that cell vulnerability to SFN-mediated apoptosis is regulated by cell cycle-dependent mechanisms (Fimognari et al., 2007). Brunelli et al. compared the chemopreventive activities of glucomoringin (GMG) and glucoraphanin (GRA), glucosinolates found in the vegetables belonging to the Brassicaceae family. They

demonstrated that both these compounds induced cell death in MM cells *in vitro* and *in vivo* mediated by cell cycle arrest and inhibition of NF-κB (Brunelli et al., 2010).

6.3.10 Head and neck squamous cancer

Head and neck squamous cell carcinoma (HNSCC) is one of the most aggressive and is the sixth leading cause of cancer-related deaths worldwide (Crowe et al., 2002). The main cause of its aggressiveness is because the malignant cells invade into local tissues and lead to the development of secondary neoplasms in about 40% of the HNSCC patients (Leon et al., 1999). Among the treatments of HNSCC, surgery, radiotherapy, and chemotherapy are the main treatments available and inspite of that, the overall 5-year survival remained <50% in the last four decades; therefore, the identification of novel drugs with promising anticancer activity is required for the intervention of HNSCC (Leon et al., 1999; Lingen et al., 2000). Various studies demonstrated that ITCs possess anticarcinogenic property against human cancers (Chung, 1992; Zhang et al., 1992; Zhang and Talalay, 1994; Stoner and Morse, 1997; Yu et al., 1998; Hecht, 1999; Rushmore and Kong, 2002). Vivian et al. reported that BITC inhibited the proliferation and induced apoptosis in HNSCC through the activation of MAP kinase pathways and cleavage of caspase 3 and poly ADP-ribose polymerase (PARP), suggesting that BITC could be used for the chemoprevention/chemotherapy of HNSCC (Lui et al., 2003).

6.3.11 Ovarian cancer

Ovarian cancer is the sixth most common and seventh leading cause of cancer-related deaths worldwide (Parkin et al., 2002). Several epidemiological and geographical studies suggest the role of dietary intake such as milk, egg, and lactose in the increased risk of ovary cancer (Kushi et al., 1999; Schulz et al., 2004). Despite advances in the screening and treatment strategies, a clear understanding in the etiology of ovarian cancer is limited (Schulz et al., 2004). Recently, interest has been directed toward the use of agents with anticarcinogenic properties including ITCs against ovarian cancer (Chang et al., 2007). In a separate report, BITC-induced growth inhibition and apoptosis in ovarian cancer cells was found to be mediated through MAP kinase pathway. BITC-mediated dose-dependent activation of JNK1/2 and p38 and downregulation of ERK1/2 and Akt expression were reported in this study. The use of specific inhibitors for JNK and p38 lead to significant decrease in cytotoxicity of BITC, suggesting that induction of apoptosis was specifically associated with activation of pro-apoptotic p38 and JNK1/2 and by inhibition of survival signals of ERK1/2 and Akt (Kalkunte et al., 2006). In another study, authors observed that antiproliferative effects of SFN were enhanced when SKOV3, C3, and T3 ovarian cancer cells were overexpressed with Akt wild-type plasmid (Chaudhuri et al., 2007). The generation of ROS induced mainly by oxidative stress has often resulted in the stimulation of cell proliferation and genetic instability in cancer cells. ITCs are known inducers of ROS, and ROS can be used in preferential killing of the cancer cells. Trachootham et al. showed that PEITC selectively killed oncogenically transformed ovarian epithelial cells via inducing ROS production mediated by the depletion of GSH antioxidant system, oxidative mitochondrial damage, and massive apoptosis (Trachootham et al., 2006). Satyan et al. demonstrated that PEITC inhibited the growth of ovarian cancer cells by causing apoptosis mediated through the activation of pro-apoptotic p38 and JNK1/2 molecules and inhibition of Akt, ERK survival pathways, and c-Myc (Satyan et al., 2006). Similarly, Pintao et al.

showed that BITC exhibit antiproliferative property against four ovarian cancer cells: SKOV-3, 41-M, CHl, and CHlcisR (Pintão et al., 1995). These studies reflect that ITCs may be useful against ovarian cancer. A very recent study from our laboratory has shown that 3,3-diiindolylmethane (DIM) from Brassica vegetables suppress the growth of SKOV-3, OVCAR-3, and TOV-21G ovarian cancer cells. The growth suppressive effect of DIM was due to G2/M cell cycle arrest. Interestingly, G2/M cell cycle arrest by DIM was associated with the remarkable activation of Chk-2 and degradation of Cdc25C. Blocking the activation of Chk-2 by transfecting the cells with DN-Chk-2 completely prevented G2/M cell cycle arrest and apoptosis by DIM (Kandala and Srivastava, 2010).

6.3.12 Skin cancer

Among the three types of skin cancer, melanoma is the most aggressive form, and one of the leading causes of death from skin cancer. It is one of the most difficult malignancies to treat. According to the National Cancer Institute's "Surveillance Epidemiology and End Results" report, approximately 68,720 people were diagnosed, out of which 8560 people died of melanoma in 2009 (Chirlaque et al., 2010; Janssen-Heijnen et al., 2010; Lasithiotakis et al., 2010; Reintgen et al., 2010; Staudt et al., 2010). The high incidence rate of melanoma could be due to (1) poor prognosis and (2) failure of the conventional therapeutic approaches used to treat melanoma (Chirlaque et al., 2010; Janssen-Heijnen et al., 2010; Lasithiotakis et al., 2010; Reintgen et al., 2010; Staudt et al., 2010). Several studies have suggested that the use of dietary ITCs and their analogs could be promising candidates for the prevention of melanoma. Manesh et al. demonstrated the effect of two naturally occurring ITCs (AITC and PITC) on the growth of B16F-10 melanoma cells. They found that both these compounds significantly inhibited the implanted B16F-10 melanoma tumor growth in C57BL/6 mice (Manesh and Kuttan, 2003). Evidence from recent reports supported the notion that BITC treatment caused disruption of mitochondrial membrane potential due to the generation of ROS, which released cytosolic apoptogenic molecules and initiated the cleavage of caspase 9, caspase 8, and caspase 3 leading to the induction of apoptosis. Kawakami et al. found that BITC treatment induced mitochondrial swelling, and cytochrome c release that were mediated through the inhibition of respiration in the mitochondria in an electrophilic reaction-dependent manner (Kawakami et al., 2005). In a similar context, it was shown that exposure of BITC to mouse skin cells resulted in the attenuation of TPA-induced oxidative damage, which was mediated through the inhibition of NADPH oxidase system that initiates the migration of leucocytes at inflamed region, supporting the fact that BITC is a potential anti-inflammatory agent (Miyoshi et al., 2004; Nakamura et al., 2004). Studies have supported the evidence that PEITC inhibits the growth of cancer cells by multiple cellular mechanisms, including induction of apoptosis associated with a rapid and transient cleavage of caspase 3 (Yu et al., 1998) or by blocking tumor promoter (TPA or EGF)-induced cell transformation in mouse epidermal JB6 cells (Huang et al., 1998). Recently, it has been shown that isoselenocyanates, a 4–6 carbon alkyl side chains with selenium substituted for sulfur in the ITCs, decreased the tumor burden of melanoma by 60% in *in vivo* model by inducing threefold more apoptosis as compared to the ITCs. This decrease in the tumor growth was associated with the inhibition of Akt survival pathway (Park and Pezzuto, 2002; Jadhav et al., 2007).

Some of the chemopreventive and chemotherapeutic properties of ITCs ranging from modulation of enzyme activity to regulation of gene expression are depicted in Figure 6.1.

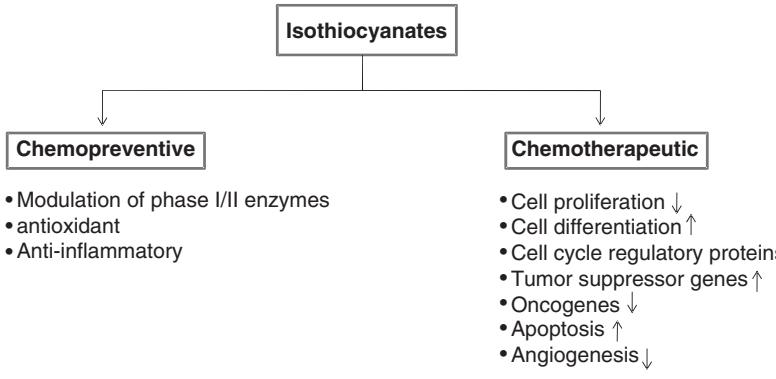

Figure 6.1. Possible chemopreventive and chemotherapeutic effect of isothiocyanates.

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7 The Disease-Preventive Potential of Some Popular and Underutilized Seeds

Rajeev Bhat

7.1 INTRODUCTION

A seed is technically defined as a "ripened plant ovule enclosing an embryo," covered by a seed coat, with an ample amount of stored food reserve. Generally, the food reserve is in the form of fat, proteins, and other bioactive compounds. Recently, the use of plant seeds and nuts has increased tremendously due to their proven beneficial effects on human health. A series of pharmacological studies has been undertaken to evaluate the potential therapeutic value of various seeds (Burits and Bucar, 2000; Zaoui et al., 2000; Bhat and Karim, 2009).

Of late, with the increased knowledge and more readily available worldwide databases on the therapeutic potential of common seeds, the search for newer seed-based food resources (indigenously present and confined to a particular location) has been the major objective of nutritionists and governmental and nongovernmental organizations linked with health care. Even though several seeds from various plant species have played a significant role as human food and animal feed, many seed resources exist that remain unexplored, possibly due to limited knowledge of their uses and/or due to the suspected presence of toxins or other antinutritional factors (ANFs). However, it has been put forward that these underutilized seeds have the potential to be a source of nutraceuticals (Sridhar and Bhat, 2007; Bhat and Sridhar, 2008; Bhat and Karim, 2009).

It is an accepted fact that, in most developing countries, nearly 80% of the general population relies on traditional medicines in primary medical care and depends on herbal pharmacotherapy, the majority of which includes seeds (Winslew and Krell, 1998; Grover and Yadav, 2004; Jung et al., 2006). In most of these cases, the scientific basis provided for the utilization of plant seeds as medicine comes from traditional knowledge confined to the local population. Seeds that are used as a medicine or for promoting better health might be in the form of oil seeds, legumes, spices, or vegetable or fruit seeds. In some instances, even some poisonous nonedible seeds are known to be useful for therapeutic purposes and exhibit high medicinal value.

Keeping the above facts in view, this chapter focuses on various types of seeds that are either used traditionally or are reported to have possible medicinal and health benefits. As

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a vast range of seeds with potential health benefits is known, only a few of the most important ones are discussed in the text, while others are provided in Table 7.1. (The health benefits of cereals and grains are not discussed, although they could be considered to be seeds in a broader sense of the definition.) The information provided should be useful for further exploration of these seeds as a potential source of pharmaceutical/therapeutic applications, and should also help to fill the existing gap in the scientific knowledge database.

7.2 OIL SEEDS AND THEIR THERAPEUTIC POTENTIAL

Most plant seeds, regardless of the plant species or variety, have lipids and/or oils as their food reserve. However, not all the oil obtained from plant seeds can be used for edible purposes or for exploration of potential medicinal uses. This is particularly due to the presence of toxic compounds, antinutrients, or the off-flavor of the extracted oil. Generally, the extraction procedure for oil also varies, involving different physical methods (cold pressing, warm pressing, ultrasound, microwave, supercritical fluid extraction) as well as chemical methods. In the following text, each of the individual and popular oil seeds (such as *Nigella*, sunflower, peanut, rapeseed, linseed, sesame, and safflower), with respect to potential health benefits, will be discussed. A detailed summary is also depicted in Table 7.1.

7.2.1 *Nigella* seeds (*Nigella sativa* L.)

This is a popular condiment generally referred to as black cumin seeds, Roman coriander seeds, black seed, and black caraway. The seeds have a pungent, bitter taste and aroma with a tinge of nutmeg. *Nigella* seeds are traditionally used in most of the Middle Eastern and Asian countries as a natural remedy for stomach disorders and for their anthelmintic, carminative, diaphoretic, diuretic, galactagogue, laxative, and stimulant properties (Chopra et al., 1986; Atia et al., 2002; Gilani et al., 2004). The seeds (or rather seed oil) contain thymoquinone, monoterpenes such as *p*-cymene and α-pinene, nigellidine, and nigellimine (El-Dakhakhny, 1963; Ansari and Sadiy, 1989; Atta et al., 1992; Atta and Hasan, 1995; Omar et al., 1999; Al-Saleh et al., 2006). For external applications, the seed is ground into a powder, mixed with sesame oil, and used to treat abscesses, hemorrhoids, and orchitis (Chopra et al., 1986). The seeds consumed with honey have been reported to exhibit protective effects against hepatotoxicity on oxidative stress and carcinogenesis (Mabrouk et al., 2002; Khadr et al., 2007; El-Kholy et al., 2009).

Reports are available wherein the volatile oil of *Nigella* has been shown to have a relaxant effect on different smooth muscles, including the aorta and jejunum, and isolated tracheal muscles in rabbits and guinea pigs (Reiter and Brandt, 1985; Aqel, 1992, 1993). Additionally, information is available wherein the seeds have been shown to encompass pharmacological properties such as hypotensive, antinociceptive, uricosuric, choleretic, antifertility, antidiabetic, and antihistaminic properties (Ali and Blunden, 2003).

7.2.2 Sunflower seed (*Helianthus annuus* L.)

Sunflower seeds are edible as such or after processing, namely, salting or roasting. The plant is widely cultivated for seed oil production. The seeds are rich in polyunsaturated

Table 7.1. Some of the common, popular, and underutilized medicinally valued seeds

Seed source (common name; Family)	Medicinal uses	Reference
Abelmoschus esculentus Moench (Okra seed: Malvaceae)	Seeds are reported to possess antioxidant properties .	Adelakun et al. (2009)
Aegle marmelos Corr. (Rutaceae)	Seeds are reported to exhibit antidiabetic and hypolipidemic effects (in diabetic rats).	Kesari et al. (2006)
Aframomum melegueta (Grains of paradise; Zingiberaceae)	Seeds possess antinociceptive action and antimicrobial and anthelmintic activities. Traditionally, the seeds are used as a purgative, galactogogue (to increase production of breast milk) and blood purifying agent. Additionally, seed extract is used to treat diarrhea.	Konning et al. (2004); Umukoro and Ashorobi (2007, 2008)
Agrimonia eupatoria L. (Rosaceae)	Seeds have been shown to have antibacterial and free radical scavenging activity.	Copland et al. (2003)
Albizzia lebbek Benth. (Siris Tree seeds, Fabaceae)	Seeds are astringent, are used for treating diabetes and piles, also have antidiarrheal activity. Pods possess antiprotozoal, hypoglycemic, antifertility, and anticancer properties.	Besra et al. (2002); Gupta et al. (2004, 2005)
Allium tuberosum (Chinese chive; Liliaceae)	Seeds are rich in unsaturated fatty acids, and possess aphrodisiac property. In traditional Chinese medicine, seeds are used for treating impotence and nocturnal emissions.	Hu et al. (2005, 2009); Sang et al. (2000a,b, 2003)
Amaranthus cruentus L. (*Amaranthus hypochondriacus* L.) (Amaranth seeds; Amaranthaceae)	Seeds are used as cholesterol-lowering agents and are rich in dietary fibers. The seed or seed oil is reported to be rich in antioxidants and useful for patients suffering from hypertension, blood pressure, cardiovascular diseases.	Lehmann (1996); Czerwiński et al. (2004); Gonor et al. (2006); Martirosyan et al. (2007)
Annona squamosa L. (Custard apple,sweet apple; Annonaceae)	Seeds possess insecticidal, antiovulatory, abortifacient, and anti-implantation properties. Seeds are also reported to show antithyroidal activity.	Vohora et al. (1975); Rao et al. (1979); Damasceno et al. (2002); Panda and Kar (2007)
Arachis hypogaea L. (groundnut/peanut) (Fabaceae)	In traditional Indian medicines, seeds have been recommended to be used as an aphrodisiac and to overcome constipation. The seed oil is reported to be useful for nephropathy and for treating dislocated joints. Isoflavones and *trans*-resveratrol from peanuts are beneficial to overcome cardiovascular diseases and cancer risks. Consumption of peanuts improves Glutathione and HDL-Cholesterol levels in experimental diabetes. Peanut is also reported to have antioxidant and anti-inflammatory (due to Arachidin-1, Arachidin-3 and isopentadienyl resveratrol) activities.	Warrier and Nambiar (1993); Sanders et al. (2000); Chang et al. (2006); Ebru et al. (2008)

Continued

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Azadirachta indica (Neem tree seeds: Meliaceae)	Seeds possess hypolipidemic and antiatherogenic effects and are reported to exhibit anthelmintic activity against gastrointestinal nematodes in sheep. Also, seed extracts is administered orally for abrogation of pregnancy in subhuman primates.	Mukherjee et al. (1996); Bopanna et al. (1997); Iqbal et al. (2010)
Barbarea verna (Mill.) Asch. (Winter cress seeds; early yellow rocket) (Brassicaceae)	Seeds reported to have anti-inflammatory activity.	Dey et al. (2006)
Bauhinia purpurea L. (Butterfly tree; Leguminosae)	Oil obtained from the seeds is a good source of essential fatty acids and lipid-soluble bioactive compounds. Seed oil also contains high amount of phytosterols.	Ramadan et al. (2006)
Brassica oleracea L. (Broccoli seeds; Cabbage seeds; Brassicaceae)	Seeds reported to have antioxidant and antigenotoxic activities. Seeds are rich in phenols and glucosinolate phytochemicals that are known to promote health (mainly prevent the stomach cancer).	Chuanphongpanich et al. (2006); Kwon et al. (2006); Rasal et al. (2006); Singh et al. (2006a); Santiago et al. (2008)
Caesalpinia bonduc L.Roxb (Nickar bean, *Caesalpinia bonducella*; Fabaceae)	Seeds are used in Indian medicine system of Ayurveda to treat tumors, cysts, and cystic fibrosis. Seeds have been shown to possess antioxidant and antimicrobial activity. Seeds possess antimicrobial activities. The seed extract have been shown to lower blood sugar in laboratory animals and cure round worm infections. After administration of the seed extracts, stress-induced animals exhibited hypoglycemia as well as depletion in serum cortisol level with increased total leukocyte count (showed adaptogenic activity).	Amarsinghe et al. (1993); Rastogi et al. (1996); Biswas et al. (1997); Sharma et al. (1997); Simin et al. (2000); Saeed and Sabir (2001); Kannur et al. (2006); Arif et al. (2009); Shukla et al. (2009)
Camellia oleifera Abel. (Tea seeds; Theaceae)	Seed oil has hepatoprotective effect and also lowering of LDL (bad cholesterol).	Lee and Yen (2006); Lee et al. (2007)
Capparis spinosa L. (Caper seeds; Capparidaceae)	Seeds contain high amount of unsaturated fatty acids, carotenoids, β-carotene, and possess antioxidant, antiproliferative, antifungal, HIV-1 reverse transcriptase inhibitory activities. Seeds are highly useful for the treatment of cirrhosis.	Hamed et al. (2007); Lam and Ng (2009); Tlill et al. (2009)
Caragana microphylla Lam. (Pea-shrub tree; Leguminosae)	Seeds have antinociceptive activity.	Huo et al. (2007)

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Carum copticum Benth. (Ajwain seeds; Apiaceae)	Seeds are used as a household remedy for treating colic diarrhea, cholera, dyspepsia, hypertension, asthma, and hepatobiliary complications. Seeds are reported to contain high amount of tannins, dietary fiber, essential oil (thymol, γ terpinene, *p*-cymene, carvacrol). Seed extracts are reported to exhibit antihypertensive, anti-inflammatory, antispasmodic, bronchodilatory, and hepatoprotective activities.	Ballba et al. (1973); Nadkarni (1986); Kapoor (1990); Uma et al. (1993); Thangam and Dhananjayan (2003); Gilani et al. (2005)
Caragana microphylla Lam. (Leguminosae)	Seed is used in Chinese folk medicine for treatment of swollen and painful throat. Seeds are reported to have antinociceptive and analgesic activities too.	Shanghai Scientific and Technical Press (1985); Jin et al. (1994); Huo et al. (2007)
Carapa guianensis Aublet. (Meliaceae)	Seed oil is used as medicinal oil for treating sprains and arthritis in parts of South America and tropical Africa. Seeds have been reported to possess anti-inflammatory properties and are used for treating coughs, convulsions, skin diseases, arthritis, rheumatism, ear infections, and to heal wounds.	Penido et al. (2006); Costa-Silva et al. (2008)
Carica papaya L. (Papaya seeds; Caricaceae)	Seeds are used for treatment against intestinal parasites in both humans and farm animals.	Robinson (1958); Krishnakumari and Majumder (1960); Bose et al. (1961); Dar et al. (1965)
Carpotroche brasiliensis (Leprosy stick seeds; Flacourtiaceae)	Seeds are reported to have anti-inflammatory and antinociceptive activities, and act as antileprotic agents.	Lima et al. (2005)
Cassia tora L. (Caesalpiniaceae)	Seed extract is reported for its hypotensive and hypolipidemic activities. Seeds are known to be liver protective.	Chan et al. (1976); Patil et al. (2004)
Cassia obtusifolia L. (Leguminosae)	Seed preparations are used in Japan, Korea, and China as a purgative to treat eye inflammation, photophobia, and lacrimation. Seeds are also reported to have neuroprotective effects.	Crawford et al. (1990); Zhu (1998); Kim et al. (2009a)

Continued

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Celastrus paniculatus Wild. (Celastraceae)	In Ayurveda, seeds and their oil are regarded as beneficial to enhance memory and protect neuronal tissues. Seeds also find its use as an appetizer, emetic expectorant, aphrodisiac, stimulant, cure for joint pain, paralysis, rheumatism, weakness. The seed oil is known to enrich the blood, cure abdominal complaints, cure cough, asthma, leprosy, and headaches. Seed powder is used externally for treating leucoderma, indolent ulcers, and scabies.	Nalini et al. (1986, 1995); Kritikar and Basu (1991); Warrier and Nambiar (1993); Borrelli et al. (2009)
Ceratonia siliqua L. (Carob seeds; Fabaceae)	Seeds have antiproliferative effects.	Corsi et al. (2002)
Chenopodium album L. and *Chenopodium quinoa* Willd. (Fat hen seeds; Chenopodiaceae)	Seeds are useful for contraceptive applications, spermicidal actions. Seeds are reported to show antioxidant and anthelmintic activities. Seeds are a good source of vitamins (like Vitamin C, thiamin, folic acid), γ-tocopherol, essential minerals (like calcium, phosphorus, magnesium iron, zinc, potassium, and copper) and unsaturated fatty acids.	Ruales and Nair (1993); Jabbar et al. (2007); Kumar et al. (2007, 2008)
Coix lachrymajobi L. (Adlay seed; Poaceae)	Seeds have been used in traditional Chinese medicine to treat tumors. Seeds have been shown to have antiproliferative effect on human lung cancer cells. Crude extract of the seed has been reported to exhibit hypolipidemic effects in obese rat fed high-fat diet. Hull extracts help in the secretion of progesterone and estradiol. The seeds' active constituents include polysaccharides like coixan A, B, and C	Takahashi et al. (1986); Numata et al. (1994); Hung and Chang (2003); Kim et al. (2004b); Huang et al. (2005); Hsia et al. (2007); Lee et al. (2008)
Coffea Arabica and C. *robusta* (Coffee seeds, Rubiacae)	Seeds are reported to have antioxidant activity and anticarcinogenic activity.	Madhava Naidu et al. (2008); Ramalakshmi et al. (2008); Ferrazzano et al. (2009)
Coriandrum sativum L. (Coriander seeds; Apiaceae/ Umbelliferae)	Seeds possess antibacterial and antioxidant activities, reduce blood pressure, and have strong lipolytic activity.	Garg and Siddiqui (1992); Leung and Foster (1996); Baratta et al. (1998)
Cuminum cyminum L. (Cumin seeds,jeera seeds; Umbelliferae)	Seed extract (oil) is used for overcoming lactagogue dyspepsia and hysteria. Seed oil is also known to have carminative and anthelmintic properties. Seed oil possesses rich antioxidant and free radical scavenging properties. Hypoglycemic effects of caraway seeds in rat models have also been reported.	Kalia et al. (1994); Foti and Ingold (2003); Yu et al. (2005); Kim et al. (2009b)

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Elaeagnus angustifolia L. (Elaeagnaceae)	Ethanolic seed extracts have been shown to encompass muscle relaxant effects that are attributed mainly to flavonoids components.	Hosseinzadeh et al. (2003)
Elettaria cardamomum (L.) Maton (Cardamon seeds; *Zingiberaceae)*	Traditionally, seeds are used as a spice, as a stimulant and carminative. Also, seeds are useful for treating teeth, oral and throat infections, congestion of the lungs, and pulmonary tuberculosis. Seeds are used to treat flatulence and improve the appetite. Seeds also have antioxidant activity.	Chopra et al. (1986); Hinneburg et al. (2006)
Eruca sativa (Synonym: *E. vesicaria* subsp. sativa (Miller) Thell., *Brassica eruca* L.) (Garden Rocket salad seeds; Brassicaceae)	Seeds possess antioxidant, antimicrobial, and renal protective activities. The seed oil has also antiseptic properties.	Barillari et al. (2005); Sarwar Alam et al. (2007); Khoobchandani et al. (2010)
Eugenia jambolana Lam. (Synonmys: *Syzygium jambolanum*, *Syzigium cumini, Eugenia cumini,* (black plum, jaman; Myrtaceae)	Seeds are used to for treating diabetic patients and have been reported to show hypoglycemic, antipyretic, and hypolipidemic effects. Seeds are rich in flavonoids and other antioxidant compounds. Seeds are also reported to show anti-inflammatory, neuropsycho-pharmacological, antibacterial, anti-HIV, and antidiarrheal effects. Seed kernel has been shown to have antihyperlipidemic effects in streptozotocin-induced diabetic rats.	Chakraborty et al. (1985a,b); Ghosh et al. (1985); Mahapatra et al. (1985); Chaudhuri et al. (1990a,b); Achrekar et al. (1991); Kusumoto et al. (1995); Bhuiyan et al. (1996); Mainzen Prince et al. (1998); Mainzen Prince and Menon (1998); Sharma et al. (2003, 2008); Ravi et al. (2005)
Fagopyrum cymosum (Trev.)Meisn. (Polygonaceae)	Lowering of blood glucose and lipid levels is observed in diabetic animals and patients on consumption of seeds.	Qin (1992); Wang (1995); Gao and You (2001)
Foeniculum vulgar L. (Fennel seeds; Apiaceae/ Umbelliferae)	The seed oil has been reported to show antioxidant, antifungal, and antimicrobial activities.	Ruberto et al. (2000); Oktay et al. (2003); Singh et al. (2006b)
Fraxinus excelsior L. (Oleaceae)	The seeds are recognized as potent hypoglycemic agents and are used for treating both type 1 and type 2 diabetes mellitus.	Eddouks and Maghrani (2004); Maghrani et al. (2004); Eddouks et al. (2005b); Visen et al. (2009)
Garcinia kola Heckel. (Bitter kola seeds, Clusiaceae)	Seeds are known to prevent gut damage and possess antioxidant and free radical scavenging activities. Also, seeds have antihepatotoxic properties, hepatoprotective effect, and the flavonoids present in the seeds exhibited hypolipidemic activity.	Iwu et al. (1987); Akintonwa and Essien (1990); Koshy et al. (2001); Okoko (2009)
Ginkgo biloba L. (Ginkgoaceae)	Seed contains antioxidant proteins, and the lipid soluble fraction of the nuts is believed to prevent cardiovascular diseases and decrease hepatic cholesterol.	Mahadevan et al. (2008); Huang et al. (2010)

Continued

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Hippophaer hamnoides L. (Sea buckthorn; Elaeagnaceae)	Seed oil is known to heal burn wounds, and the oil is reported to have therapeutic role in atopic dermatitis, cardiovascular diseases, and gastric ulcers. Seed oil is rich in bioactive substances like carotenoids, tocopherols, omega-3, omega 6-fattyacids, and phytosterols.	Yang et al. (1999); Johansson et al. (2000); Eccleston et al. (2002); Xing et al. (2002); Basu et al. (2007); Upadhyay et al. (2009)
Holarrhena antidysenterica (Apocynaceae)	Seeds are used as an antidiabetic drug in Asian countries. Seeds have been proved to exhibit antidiabetic, antihyperlipidemic, and antimicrobial activities.	Jolly and Mechery (1996); Wahab and Yousuf (2004); Pankaj et al. (2005); Ali et al. (2009)
Hunteria umbellata K. Schum. (Apocynaceae)	Seeds have hypoglycemic and antihyperlipidemic effects.	Adeneye and Adeyemi (2009); Adeneye et al. (2010, 2011)
Juglans nigra L. (Walnut; Juglandaceae)	Seeds (nut) is reported to have antioxidant and antibacterial activities. Walnut contains an antioxidant compound (ellagic acid) that supports the immune system and appears to have several anticancer properties. In traditional Chinese medicine, walnut seeds were considered as a kidney tonic and good for the brain, back, and skin.	Fukuda et al. (2003); Pereira et al. (2007); Labuckas et al. (2008)
Khaya senegalensis (Desr.) A. Juss (Meliaceae)	Seeds have antimicrobial activity. The oil obtained from the seeds is used as emmenegogue. Seeds contain bioactive compounds of potential therapeutic and prophylactic significance. In Nigeria, traditionally, the seed oil is used to treat diabetes mellitus. Seed extracts have been reported to show anthelmintic activity against gastrointestinal nematodes of sheep.	Dalziel (1956); Watt and Breyer-Brandwijk (1962); Ademola et al. (2004); Audu-Peter et al. (2006); Ayo et al. (2007)
Lactuca sativa L. (Lettuce; Asteraceae)	The seeds are traditionally used in Iran for relieving inflammation, gastrodynia, and osteodynia. Seeds have also been reported to exhibit analgesic and anti-inflammatory activity in rats.	Aqili Khorasani (1991); Sayyah et al. (2004)
Lepidium sativum L. (garden cress seeds; Cruciferae)	Seeds have healing properties and are applied as a poultice for wounds and sprains, show diuretic effects, are aperient, demulcent, aphrodisiac, carminative, galactagogue, and emmenagogue, rubefacient. The seeds indicate the presence of cardioactive substance and are shown to have probable action through adrenergic mechanisms. The aqueous extract of seeds has been reported to exhibit a potent hypoglycemic activity in normal and streptozotocin-induced diabetic rats.	Nadkarni (1954); Vohora and Khan (1977); Chopra et al. (1986); Eddouks et al. (2005a); Patel et al. (2009)

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Linum usitatissimum L. (Linseed; Linaceae)	Seed oil is rich in omega-3 fatty acids and is reported to be beneficial for preventing cardiovascular problems, heart disease, arrhythmia, cancer, and for reducing inflammation leading to atherosclerosis.	Mest et al. (1983); Tolkachev and Zhuchenko (2000); Muir and Westcott (2003); Chauhan et al. (2009)
Litchi chinensis Sonn. (Sapindaceae)	Seed aqueous extract has been reported to have hypoglycemic effects that were equivalent to glibenclamide and phenformin.	Kuang et al. (1997)
Livistona chinensis (Jacq.) R. Br. ex Mart. (Chinese Fan Palm; Arecacea/Palmae)	Seeds have been shown to possess antiproliferative, antibacterial, and antiangiogenic properties. Seeds are reported to be effective against human cancer (breast and colon). Traditional Chinese medicine uses the seeds as an analgesic, hemostatic, and for treating esophageal cancer.	Sartippour et al. (2001); Kaur and Singh (2008a,b); Wang et al. (2008)
Lonchocarpus sericeus (Poir.) Kunth (Lilac tree seeds; Fabaceae)	Seeds show anti-inflammatory and antimicrobial effects.	Alencar et al. (2005); Rai et al. (2006)
Lupin species (Fabaceae)	Seeds help in the modulation of blood glucose, possess antioxidant activity, have high amount of phenols (like isoflavones, flavones, and dihydroflavonols) and helps in cholesterol metabolism.	Chango et al. (1999); Dueñas et al. (2009); Martínez-Villaluenga et al. (2009); Ranilla et al. (2009); Terruzzi et al. (2011)
Macrotyloma uniflorum (Lam.) Verdc. (Fabaceae)	Seeds are reported to have antioxidant and free radical scavenging activities.	Siddhuraju and Manian (2007)
Mangifera indica Linn. (Anacardiaceae)	In Indian traditional medicine, seeds have been recommended to be used to cure vomiting, dysentery, and burning sensation in heart. Decoction of the kernel and seed is prescribed for diarrhea.	Krithikar and Basu (1984); Sairam et al. (2003)
Momordica cochinchinensis (Lour). (Cucurbitaceae)	Seeds are reported to have antioxidative effects. In traditional Chinese medicine, seeds are believed to have cooling properties, and are used for liver and spleen disorders, treating wounds, hemorrhoids, bruises, and swelling. The seed membrane is reported to be used to make a tonic for children and lactating or pregnant women, and to treat "dry eyes" (xerophthalmia) and night blindness.	Guichard and Bui (1941); De Shan et al. (2001); Tsoi et al. (2005)
Momordica charantia L. (Bitter melon; Cucurbitaceae)	Seed caused significant reduction in fasting blood glucose and improved glucose tolerance in normal and diabetic animals and humans. Seeds are reported to retard retinopathy in alloxan-induced diabetic rats.	Srivastava et al. (1987, 1993); Raman and Lau (1996); Ahmed et al. (2001); Fan and Cui (2001); Miura et al. (2001)

Continued

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Moringa olifera Lam. (horseradish tree; Moringaceae)	Seeds are reported to prevent oxidative stress, seed extract can act against CCl_4-induced liver injury and fibrosis in rats. Seed extracts are reported to have hepatoprotective effects against Diclofenac (anti-inflammatory drug that causes liver toxicity)-induced hepatotoxicity in rats. Seeds also have larvisidal activity against *Aedes aegypti*.	Hamza (2007, 2010); Ferreira et al. (2009)
Nelumbo nucifera Gaertn. (Lotus seeds; Nympheaeceae)	Seeds possess antioxidant, antiaging and antimicrobial properties, and are reported to enhance immunity. Seeds are also used to treat cancer, diuretics, tissue inflammation, skin diseases, and are generally prescribed to children as diuretic and refrigerant. Seed powder mixed with honey is used for treating cough.	Liu et al. (2004); Yen et al. (2005); Sridhar and Bhat (2007)
Nigella sativa L. (black seed, black cumin, black caraway) (Ranunculaceae)	Seeds are highly medicinal and are used to treat headache, migraine attacks, coughs, asthma, abdominal pain, flatulence, diarrhea, and rheumatism. Aqueous and oil extracts of the seeds are reported to possess antioxidant, anti-inflammatory, immunomodulatory, anticancer, analgesic, and antimicrobial activities.	Hanafy and Hatem (1991); Burits and Bucar (2000); Zaoui et al. (2000); Kumara and Huat (2001); El-Abhar et al. (2003); Parker et al. (2003); Yu et al. (2005); Gali-Muhtasib et al. (2006); Kanter et al. (2006); Salih et al. (2009); Shahzad et al. (2009)
Paullinia cupana Kunth. (Guarana seeds; Sapindaceae)	Seeds are reported to have antiplatelet, antioxidant, and antibacterial activities, and have inhibitory effects on hepatocarcinogenesis in mice. Guarana seeds are also recommended for therapeutic purposes like antidiarrheic, diuretic, antineuralagic agent, as painkiller, febrifuge, and to treat migraine.	Henman (1982); Seidemann (1998); Basile et al. (2005); Fukumasu et al. (2006); Majhenič et al. (2007); Subbiah and Yunker (2008)
Ocimum sanctum Linn. (Basil, Sacred Basil, Holy Basil, Tulsi; Lamiaceae)	Oil from seeds possesses significant anti-inflammatory, antipyretic, analgesic, immunomodulatory effects, and antiarthritic activities.	Singh and Majumdar (1995a,b, 1997); Singh et al. (1996); Mediratta et al. (2002)
Opuntia ficus-indica (L.) Mill. (Prickly pear seed; Cactaceae)	Seeds exhibit hypoglycemic and hypocholesterolemic effects, attributed to the presence of unsaturated fatty acids.	Ennouri et al. (2006a,b, 2007)
Picralima nitida (Stapf) Th.&H.Dur. (Apocynaceae)	Antiplasmodial effects, pseudo-akuammigine, an alkaloid from extract, is reported to possess anti-inflammatory and analgesic actions	Duwiejua et al. (2002); Okokon et al. (2007)

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Pimpinella anisum L. (Umbelliferae)	In Turkish folk medicine, seeds are used as appetizer, tranquillizer, and as a diuretic. Seeds also possess antioxidant and antimicrobial activities.	Gulçina et al. (2003)
Plantago asiatica L. (Plantaginaceae)	Seeds can enhance the immune function of the immunosuppressant mice and have immune modulatory effect.	Xie et al. (2006); Huang et al. (2009)
Psidium guajava L. (Guava seeds; Myrtaceae)	Seeds contain high phenolics and antioxidant compounds. Also, antibacterial glycine-rich peptide from the seeds has been isolated.	Pelegrini et al. (2008); Castro-Vargas et al. (2010)
Psoralea corylifolia L. (Fabaceae)	Seeds are used in Ayurvedic medicines as stomachic, deobstruent, anthelmintic, diuretic, for curing skin disease like that of leucoderma and leprosy. Seeds are also reported to have cytotoxic, anticancer, and immunomodulatory properties.	Kotiyal and Sharma (1992); Latha and Panikkar (1998)
Picralima nitida (Stapf) Th.&H.Dur. (Apocynaceae)	Antiplasmodial effects, pseudo-akuammigine, an alkaloid from extract, is reported to possess anti-inflammatory and analgesic actions	Duwiejua et al. (2002); Okokon et al. (2007)
Prunus amygdalus Batsch. (Almond seeds; Rosaceae)	Seed oil contains high amount of unsaturated fatty acids. Seeds have cholesterol lowering effects, are used for treating diabetes and glycosuria patients and are reported to exhibit high antioxidant and anticancer properties. In Indian traditional medicines, seeds are used as a nutritive source for brain and improvising the nervous system.	Spiller et al. (1998); Davis and Iwahashi (2001); Josse et al. (2007)
Pterodon pubescens Benth. (Leguminosae)	Seeds have antirheumatic, analgesic, and anti-inflammatory activities, while its oil has been demonstrated to have cercaricidal activity.	Mors et al. (1967); Sabino et al. (1999)
Ribes nigrum L. (Blackcurrant seeds; Grossulariaceae)	Seeds are reported to show antioxidant properties. The seed oil is rich in vitamin E and unsaturated fatty acids like omega-6 fatty acid, gamma-linolenic acid, omega-3 fatty acid, and alpha-linolenic acid. Seed oil is used for treatment of canine atopic dermatitis.	Traitler et al. (1984); Samotyja and Małecka (2007)
Rhynchosia volubilis Lour. (Fabaceae/ Leguminosae)	Seeds contain high amount of isoflavone that function as partial agonists or antagonists of estrogen. Seed is also known to prevent postmenopausal osteoporosis.	Kim et al. (2005)
Salvia hispanica L. (Chia seeds; Lamiaceae)	Seed oil has antidiabetic effects, have high levels of antioxidants, dietary fiber, inhibits growth and metastasis in this tumor model.	Ayerza and Coates (2005); Espada et al. (2007); Reyes-Caudillo et al. (2008)

Continued

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Securigera securidaca L. (Fabaceae; Leguminosae)	In Iranian folk medicine, seeds are used for treatment of hyperlipidemia, diabetes, and epilepsy. Seeds are reported to exert marked chronotropic, diuretic, and hypokalamic activities. Seeds are also reported to decrease lipid levels and peroxidation, and improve vascular endothelium-dependent relaxation in hypercholesterolemia.	Al-Hachim and Maki (1969); Ali et al. (1998); Hosseinzadeh et al. (2002); Garjani et al. (2009)
Sinapis alba L (Synonym: *Brassica alba*) (Mustard seeds; Brassicaceae)	Seeds are used as spice. In India, traditionally, seed oil is applied for relieving pain. Seed contains Myrosinase, an enzyme found in all glucosinolate-containing plants, responsible for the conversion of glucosinolates into products that is beneficial to health.	Rastogi et al. (2004); Van Eylen et al. (2006)
Solanum torvum L. (Turkey berry pea eggplant; Solanaceae)	Novel protein isolated from the seeds showed excellent antioxidant activity.	Sivapriya and Srinivas (2007)
Sophora japonica L. (Leguminosae)	Seeds are known to contain high amount of flavonoids. Seeds have abortifacient, emetic, hemostatic, antibacterial, antispasmodic, anticholesterolemic, anti-inflammatory, diuretic, emetic, emollient, febrifuge, hypotensive, purgative, styptic, and tonic properties.	Duke and Ayensu (1985); Tang et al. (2001)
Strychnos nux-vomica L. (Loganiaceae)	Improves blood circulation and relieves rheumatic pain, cleans the fine hairs that cause throat irritation. Seeds are also reported to have analgesic and anti-inflammatory activities.	Wu et al. (1994); Guizhi (1996); Yin et al. (2003)
Strychnos potatorum Linn. (Loganiaceae)	Seeds are known to be useful in hepatopathy, nephropathy, gastropathy, bronchitis, chronic diarrhea, dysentery, diabetes, and eye diseases. Seeds are also known to possess antiulcerogenic potential.	Asima and Satyesh (2001); Sanmugapriya and Venkataraman (2006, 2007)
Tamarindus indica L. (Tamarind seed; Leguminosae)	Seed extracts possess antidiabetic and antihyperlipidemic activity. Seeds are also rich in antioxidant compounds.	Maiti et al. (2004, 2005); Siddhuraju (2007)
Trigonella foenum-graecum Linn. (Fenugreek seeds; Fabaceae)	Seeds are shown to have antioxidant and free radicals scavenging activities. Seeds also showed hypolipidemic activity and anti-atherosclerotic effects. Seeds are used for treating various gastrointestinal disorders, preventing ulcer and for controlling diabetic sugar level. Seeds are reported to have potential antifertility activity in rabbits.	Sharma and Raghuram (1990); Puri (1998); Suja Pandian et al. (2002); Kassem et al. (2006); Kaviarasan et al. (2007)
Thevetia peruviana (Pers.) Schumann. (Yellow oleander; Apocynaceae)	Seeds are reported to have antifungal activity (but seeds are highly poisonous).	Gata-Gonçalves et al. (2003)

Table 7.1. *Continued*

Seed source (common name; Family)	Medicinal uses	Reference
Urtica dioica L. (Urticaceae)	Seeds showed the presence of high amount of PUFA. The seeds possess hepatoprotective and antioxidant properties. In Turkey, seeds are used as herbal medicine for treating cancer patients.	Akbay et al. (2003); Gozum et al. (2003); Guil-Guerrero et al. (2003); Yener et al. (2009)
Vaccaria segetalis (Neck.) Carcke (Caryophyllaceae)	In traditional Chinese medicine, seeds are widely used to promote diuresis and milk secretion, activate blood circulation, and relieve carbuncle.	Sang et al. (2003a)
Vernonia anthelmintica (L) Willd. (*Centratherum anthelminticum* L., *Conyza anthelmintica*) (Asteraceae)	Seeds possess anthelmintic, antifilarial, antidiabetic, and antihyperlipidemic activities.	Iqbal et al. (2006); Fatima et al. (2010)
Vigna aconitifolia Jacq. (Moth bean; Fabaceae)	Seeds are rich in polyphenols and other antioxidant compounds.	Siddhuraju (2006)
Vitis vinifera L. (Grape seeds; Vitaceae)	Seeds are a good source of proanthocyanidins and other antioxidant compounds and have antiulcer, antiproliferative, and antimicrobial properties.	Tebib et al. (1997); Saito et al. (1998); Bagchi et al. (1998, 2000); Cho and Lee (2009); Furiga et al. (2009)
Ziziphus mauritiana Lamk.(Rhamnaceae)	Seed extracts has been reported to exhibit immunostimulatory potential.	Mishra and Bhatia (2009)

fatty acids with low saturated fats, are a good source of dietary fiber, vitamin E, minerals, and protein, and have demonstrated high antioxidant and antimicrobial activities (Kato et al., 1997; Bailly et al., 2000; Rodrigues et al., 2004; Giada, 2008; Mendoza et al., 2008).

7.2.3 Groundnut seed (*Arachis hypogea* L.)

This seed is popularly known as the peanut, monkey nut, or earth nut and is widely cultivated for edible purposes (it is high in protein, energy, vitamins, and minerals) as well as for its oil. The seeds are sometimes used as a replacement for almonds and are also used in the preparation of peanut milk (a lactose-free milk-like beverage), peanut butter, and gluten-free flour. Peanut oil is exclusively used for cooking, has a mild flavor, is resistant to rancidity, and is stable even at high cooking temperatures.

Peanuts are rich in nutritious antioxidant compounds, which are a good source of dietary fiber and have niacin (which contributes to brain health and blood flow), vitamin E, manganese, and phosphorus. The seed and seed oil are free of *trans* fats and sodium. The seeds are reported to be a good source of resveratrol, which is known to possess significant anti-aging effects and to reduce cardiovascular disease and risks of cancer (Sanders et al., 2000). In traditional Indian medicine, the seeds are recommended to be used as an aphrodisiac and to overcome constipation, and the seed oil is recommended to be used for nephropathy and for treating dislocated joints. Peanut consumption is reported to improve glutathione

and high-density lipoprotein (HDL) cholesterol levels in experimental diabetes tests (Warrier and Nambiar, 1993; Chang et al., 2006; Ebru et al., 2008).

7.2.4 Sesame seeds (*Sesamum indicum* L.)

Sesame seeds are rich in oil and contain vital minerals (such as manganese, copper, calcium, iron, and magnesium), vitamins (thiamine and tocopherol), and phytosterols. To date, three varieties of sesame seeds are known: white, black, and red. Among these, the black seed variety has been widely used and recommended for medicinal purposes. All these varieties are grown for their use in food and as a source of gingelly oil (sometimes used as a substitute for olive oil). Sesame seeds have emollient, demulcent, emmenagogue (stimulating menstruation), laxative, diuretic, and fattening properties. The seeds contain two important lignans: sesamin and sesamolin. Sesamin can act as a phytoestrogen and has bactericide and insecticide activities, as well as having antioxidant activity and health-promoting benefits (Kato et al., 1998). The seeds are recognized as having cholesterol-lowering effects, and can reduce inflammation and pain associated with rheumatoid arthritis. The seeds are also useful for treating respiratory disorders, for treating external skin disorders (such as ulcers, burns, and scalds), and as a remedy for piles. Sesame seed consumption is reported to enhance the plasma gamma-tocopherol and vitamin E activity, which are capable of preventing cancer and heart disease (Cooney et al., 2001).

Sesame seed oil is used for improving oral health as an antibacterial mouthwash. Sesame oil is also used as a solvent for intramuscular injections (Tyler et al., 1976). Smith and Salerno (2001) reported the oil to contain linoleate in triglyceride form that can selectively inhibit the malignant melanoma growth. However, the only impediment to extensive use of this seed is the presence of a high amount of antinutrients (such as phytic acid) and oxalic acid.

7.2.5 Oilseed rape (*Brassica napus* L.)

This is commonly referred to as rapeseed oil or canola oil. The oil has a high content of erucic acid and low glucosinolate content, which renders it of nutritionally low significance. Canola oil is recognized for its health benefits due to the presence of high amounts of both omega-6 and omega-3 fatty acids (linoleic acid and linolenic acid) (Barth, 2009; Vollman and Rajcan, 2009).

7.2.6 Safflower (*Carthamus tinctorius* L.)

Safflower seeds are primarily cultivated for the production of edible cooking oil. The oil is flavorless, rich in polyunsaturated fatty acid (PUFA), and comparable to sunflower seed oil. The major portion of safflower oil contains serotonin derivatives that have been proven to show strong *in vitro* antioxidative activities and to exert various biological effects on plasma and liver lipid status, ischemia-reperfused Langendorff hearts, and cellular proinflammatory cytokine or melanin production (Sakamura et al., 1978; Nagatsu et al., 2000; Hotta et al., 2002; Cho et al., 2004). In Korea, safflower seed oil is used as an herbal medicine for the promotion of bone formation and treatment of osteoporosis and rheumatism. Investigations on the effects of phospholipids from safflower seeds have shown decreased hepatic lipid levels via decreased liver cholesterol and increased fecal neutral steroids (Cohn et al., 2008). Koyama et al. (2006) reported the ethanol-ethyl acetate extract of

defatted safflower seeds to be preventive against atherosclerotic development, and its major and unique subclass of polyphenols, that is, serotonin derivatives, to be responsible for its antioxidative and antiatherosclerotic activities.

7.2.7 Linseed (*Linum usitatissimum* L.)

The oil obtained from the linseed plant is popularly known as flaxseed oil. The seeds contain high amount of alpha-linoleic acid content (C18:3, n-3), and the oil obtained from the dried ripened seeds is reported to contain high levels of omega-3 fatty acids (Muir and Westcott, 2003). Linseed oil is prescribed in Indian traditional medicine to relieve constipation and as a cure for skin inflammation. The seed oil is known to be beneficial for preventing cardiovascular problems, arrhythmia, and cancer, and for reducing inflammation that leads to atherosclerosis (Mest et al., 1983; Tolkachev and Zhuchenko, 2000; Chauhan et al., 2009). Daily food supplementation with ground linseed and linseed oil has been reported to have a preventive effect against cardiovascular diseases (Tarpila et al., 2002). Additionally, linseed oil alone or in combination with other oils is reported to have antimicrobial activities (Gur et al., 2006).

7.3 SPICE SEEDS AS MEDICINE

Spices, a broader category than seeds, can be defined as a plant product (usually in dry form) that is used in small quantities as a food additive to impart flavor during food preparation. Most of the time, spices, being natural and of plant origin, contain several bioactive compounds that can exert potential health benefits, even at threshold levels. In the following text, only a few spices that are available in the form of seeds are discussed.

7.3.1 Coriander seeds (*Coriandrum satium* L.)

Coriander seeds are small, ovoid seeds with a mild, sweet, pungent, citrus-like aroma with a hint of sage, mainly due to the presence of high amounts of essential oils. The seeds are extensively used as a seasoning agent or as an ingredient in curry powder, cakes, breads, pastries, and alcoholic beverages in parts of India, China, and the Middle East. Due to their intense aroma, the seeds also find a place in aromatherapy (Cooksley, 2003). Coriander seeds are used medicinally for overcoming indigestion, rheumatism, and pain in the joints, and for treatment of worms or intestinal parasites (Wichtl, 1994). Available reports indicate seed volatiles (essential oil) to possess antibacterial and antioxidant activities (Garg and Siddiqui, 1992; Baratta et al., 1998; Tanabe et al., 2002). The antioxidative effect of coriander seeds against hexachlorocyclohexane (HCH)-induced formation of free radicals in rat livers has been reported by Anilakumar et al. (2001). Studies have indicated that coriander seeds display hypoglycemic action and affect the carbohydrate metabolism (Gray and Flatt, 1999; Chithra and Leelamma, 2000). Vasudevan et al. (2000) reported the seeds to exhibit gastric acid secretion by a cholinergic mechanism. Coriander seeds are also known to reduce blood pressure in laboratory animals (Medhin et al., 1986) and exhibit strong lipolytic activities (Leung and Foster, 1996). Recent reports have also indicated coriander seeds to cause diuresis in rats (Aissaoui et al., 2008).

7.3.2 Caraway (*Cumin carvi* L.)

Caraway seeds, popularly known as fennel seeds, contain high amounts of etherics (essential oils) and are reported to have high medicinal value (De Carvalho and Da Fonseca 2006; Lemhadri et al., 2006; Mazaki et al., 2006). Traditionally, in most parts of Asia, seed extracts (oil) are used for overcoming lactagogue dyspepsia and hysteria, as well as for their carminative and anthelmintic properties. Carvone, a chemical compound isolated from caraway oil, is believed to be responsible for the anthelmintic properties (useful against hookworms present in the intestines) and for overcoming flatulence and stomach disorders. The seed oil has also been reported to possess rich antioxidant and free radical scavenging activities (Foti and Ingold, 2003; Yu et al., 2005). Strong hypoglycemic effects of caraway seeds in rat models have also been reported (Kalia et al., 1994).

7.3.3 Pepper seeds (*Piper nigrum* L.)

Seeds of black pepper are produced from green, unripe berries and are widely used as a spice and condiment. Black pepper seeds are known to possess both pharmacological and toxicological properties. The seeds are known to improve appetite and to enhance digestive power and are used to treat cold, cough, dyspnea, throat infection, dysentery, indigestion, diarrhea, flatulence, intestinal worms, epilepsy, piles, elephantiasis, and filaria. Pepper seeds exhibit high antimicrobial activity, and the presence of two phenolic compounds, namely 3,4 dihydroxy phenyl ethanol glucoside and 3,4-dihydroxy-6-(*N*-ethylamino) benzamide, is reported to be responsible for black pepper's antibacterial properties (Bandyopadhyay et al., 1990; Venkat Reddy et al., 2004). Pepper seeds are also reported to have analgesic, antipyretic, antioxidant, anti-inflammatory, and anticarcinogenic properties (Unnikrishnan and Kuttan, 1990; Nalini et al., 1998; 2006; Saxena et al., 2007; Duessel et al., 2008). Piperine, which is known to be responsible for the pungent aroma it imparts to pepper, is recognized as the contributing factor for pepper's medicinal benefits. Piperine has been shown to have a protective impact on liver enzymes, to inhibit drug-metabolizing enzymes in rodents, and to increase plasma concentrations of several drugs, including P-glycoprotein substrates (phenytoin and rifampin) in humans (Bhardwaj et al., 2002). A significant increase in the absorption of selenium, vitamin B, beta-carotene, and curcumin is reported due to the presence of piperine (Duke and DuCellier, 1993; Mittal and Gupta, 2000; Ravindran, 2000).

7.3.4 Cumin seeds (*Cuminum cyminum* L.)

Cumin seeds are one of the common household spices generally used as a flavoring agent for seasoning purposes, particularly in the preparation of curries, soups, and bakery products. Traditionally, cumin seeds are used as a stimulant and are known for their antispasmodic, diuretic, emmanogogic, and carminative properties (Chopra et al., 1958; Behera et al., 2004). In the Indian system of Ayurvedic medicine, cumin seeds are used to treat dyspepsia, diarrhea, and jaundice.

Cumin seeds are rich in volatile oils, which impart the characteristic aroma to the seed. The seeds have antioxidant properties (Kim et al., 2009b) and show antihydrolytic effects that are considered to be comparable to those of conventional synthetic antioxidants (The Wealth of India, 2001). Earlier, a dietary regimen containing cumin powder (1.25%) was reported to be remarkably beneficial, as indicated by reduction in hyperglycaemia and

glucosuria along with lowered blood urea level and reduced excretions of urea and creatinine in diabetic animals (Willatgamuwa et al., 1998).

7.3.5 Fenugreek seeds (*Trigonella foenum*-graecum L.)

These seeds are used as a spice as well as an herbal medicine in most parts of Asia for treating metabolic and nutritive dysfunctions and diabetes. Fenugreek seeds possess high amounts of nutritive properties, are known to stimulate digestive processes, and are used extensively for treating various gastrointestinal disorders (Puri, 1998). The effects of fenugreek seeds on hypercholesterolemic/hypolipidemic activities have been reported (Sharma et al., 1996). Suja Pandian et al. (2002) reported that aqueous extract and a gel fraction isolated from the seeds exhibited significant ulcer-protective effects in rats. A significant decrease in the insulin/glucose ratio in subjects administered repeatedly with fenugreek seed extract, along with decreased dietary fat consumption in otherwise healthy overweight subjects, has been reported by Chevassus et al. (2009).

7.4 LEGUMES AND MEDICINAL USE

From time immemorial, legumes (Fabaceae/Leguminosae) comprising both common and wild species have played a significant role as a part of traditional diets in most of the indigenous populations around the world. Legumes, being low in fat, are a good source of protein, dietary fiber, minerals, several bioactive compounds, and phytochemicals (Anderson et al., 1999; Bhat and Karim, 2009). The consumption of various types of legumes is known to reduce the risk of diabetes and obesity and is known to have an inhibitory role in the reduction of coronary heart diseases (Bazzano et al., 2001). Recent reports indicate that legume seeds encompass high amounts of polyphenolic compounds with potential antioxidant activities (Tsuda et al., 1994; Mazur et al., 1998; Shahidi et al., 2001; Bhat and Karim, 2009). As several health benefits of commonly consumed legumes (such as cowpeas, kidney beans, and fava beans) are already known, in the following text, only the soybean and a few underutilized legumes are discussed.

7.4.1 Soybeans (*Glycine max* (L.) Merrill)

The soybean is one of the most popular legumes and is highly versatile both nutritionally and medicinally. Regular consumption of soybeans or their products is known to overcome or prevent several degenerative and chronic diseases, including cancer, especially among Asians (Lee et al., 1991; Barnes et al., 1994; Messina et al., 1994). Soy intake has been reported to have protective effects against breast, prostate, endometrial, and colon cancer (Adlercreutz, 1995; Goodman et al., 1997; Kurzer and Xu, 1997; Nagata et al., 1997). Consumption of tofu has been related to reduced risk of breast cancer (Wu et al., 1996). Soybeans are rich in isoflavones (Barnes et al., 1994) whose cancer protective characteristics are believed to be based on their antioxidant, antiestrogenic, antimutagenic and antiproliferative, differentiation-inducing, and apoptosis-inducing effects (Peterson and Barnes, 1993; Cassidy et al., 1994; Constantinou and Huberman, 1995; Makela et al., 1995; Wei et al., 1995; Kyle et al., 1997; Franke et al., 1998).

Some of the underutilized legume seeds, such as *Mucuna* sp., *Sesbania* sp., *Canavalia* sp., and *Abrus precatorius* have been used traditionally for treating various diseases, with

reports available to support this scientifically. The dried seeds of legume *Abrus precatorius* L. are traditionally consumed by certain African tribes as a treatment for diabetes and chronic nephritis, which has been proven scientifically in animal models (Monago and Alumanah, 2005). The seed powder is also used as an oral contraceptive by various African tribes (Watt and Breyer-Brandwijk, 1962). In Indian traditional medicine, the seed powder is used to treat asthma.

The seed flour of *Sesbania* sp. is used in the treatment of various skin diseases, ringworm infections, and wounds (Duke, 1981). *Canavalia gladiata* seeds are used for curing skin rashes in Chinese medicine (Kay, 1979), while fermented *C. gladiata* seed has been reported to reduce the viability of liver cancer cells (HepG2 by 80%) (Chen et al., 2000). Seeds of *Canavalia ensiformis* possess anticancerous properties and are cytotoxic to human pancreatic cancer cells (Swaffer et al., 1995; Morris, 1999).

7.4.2 *Mucuna pruriens* L.

Mucuna pruriens L., another underutilized, nutraceutically valued legume, is known to contain high amounts of L-dopa (3,4-dihydroxy-L-phenylalanine), which is useful in treating Parkinson's disease; the effect is known to be equivalent to that of synthetic L-dopa (Prakash and Tewari, 1999; Bhat et al., 2007). The seed powder of *Mucuna* is also used for treating joint pain, overcoming irregular menstruation, and for treatment of diabetes, leucorrhea, and spermatorrhea (Nadkarni, 1982; Ding et al., 1991). Reports are also available wherein decoctions of *Mucuna* seeds have shown cholesterol and lipid-lowering effects in plasma in rats (Iauk et al., 1989). *Mucuna* seeds also exhibit high antioxidant and antimicrobial activity (Tripathi and Upadhyay, 2001; Rajeshwar et al., 2005).

7.4.3 Tamarind seeds (*Tamaridus indica* L.)

The tamarind tree grows naturally in tropical and subtropical regions of the world. Generally, the pulp portion is used as a flavor agent, particularly in the preparation of curries and sauces. In certain instances, the flower and leaf are eaten as vegetables. In some parts of India, the seeds are roasted and eaten and the seed's powdery coating is used as a coffee substitute. Traditionally, dried tamarind pulp and seeds are used for preventing the rancidity (lipid peroxidation) and for enhancing the shelf life of peanut oil and coconut oil (Siddhuraju, 2007).

The aqueous extract of tamarind seeds is reported to show antidiabetic and antihyperlipidemic activities, which can reduce blood sugar levels, total cholesterol, and triglycerides (in streptozotocin-induced diabetic male rats) (Maiti et al., 2004, 2005). Tamarind seeds are also known to show potential antioxidant activity, wherein the isolated antioxidant components include 2-hydroxy-30,40-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate,3,4-dihydroxyphenylacetate, and (-)-epicatechin, in addition to oligomeric proanthocyanidins (Tsuda et al., 1993, 1994, 1995). According to Tsuda et al. (1994), the seeds are a safe and low-cost source of antioxidants, and the ethanol extract prepared from the seed coat exhibits potential antioxidative activity as measured by the thiocyanate and thiobarbituric acid (TBA) method.

Siddhuraju (2007) reported antioxidant properties of polyphenolic compounds extracted from defatted raw and dry-heated tamarind seed coat. Suksomtip and Pongsamart (2008), in their studies on Thai tamarind seeds, reported methanolic extracts from the seed coat to have a protective effect against oxidative stress of biomolecules.

7.5 UNDERUTILIZED SEEDS

It is difficult to categorize whether a seed is underutilized or not. However, considering wide usage and based on the popularity of an individual seed species in the preceding text, an attempt has been made to identify a few of the plant seeds with potential health-promoting activities that could be explored further. Detailed descriptions of a few of the unpopular/underutilized seeds are summarized in Table 7.1.

7.5.1 Perilla (*Perilla frutescens* [Hassk.])

Perilla seeds, which are grayish-brown to yellowish-white with purple striations in addition to a slightly pungent aroma, form one of the most important oilseed crops that have been widely preferred and consumed for their edible vegetable oil, as a spice, and as a medicine, especially in Asian countries (Korea, China, India, and Japan). Traditionally, the seeds are used to treat coughs and phlegm obstructions and to aid in cases of constipation. The seeds are rich in PUFA and have rich antioxidant and (colon) cancer preventive properties (Narisawa et al., 1994; Nagatsu et al., 1995). In animal models (rats), perilla oil is reported to prevent the excessive growth of visceral adipose tissue (Okuno et al., 1997). The seed oil is composed mainly of fatty acids, such as palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and R-linolenic acid (18:3) (Longvah and Deosthale, 1991; Kwak, 1994; Lee et al., 2002). Gamma-linolenic acid (GLA), an *n*-3 unsaturated fatty acid and a precursor of long-chain *n*-3 PUFA, is present in higher amounts (up to 60%), followed by oleic and linoleic acid. ALA has been reported to have beneficial effects in the control of chronic disease, and intake of perilla oil is known to lower the plasma lipid level of rats (Connor, 2000; Kim et al., 2004a). Adhikari et al. (2006) studied the policosanols (a mixture of long-chained primary alcohols used as nutritional supplements and known to reduce platelet aggregation, endothelial damage, cholesterol levels, and foam cell formation) in perilla seeds and found that the policosanols exist in free or nonesterified alcohols similar to those in grain sorghum. This study indicated that consumption of perilla seeds might be beneficial in supplying policosanols to the diet.

7.5.2 *Hunteria umbellata* ([K. Schum] Hallier f.)

In African traditional medicine, a water decoction made from the dry seeds of *Hunteria* (Apocynaceae) is highly valued in controlling diabetes mellitus. In this treatment, an average of 2–3 tsp (10–15 g/day) of the pulverized plant seed soaked in a glass full of hot water (20–30 minutes) is given for oral intake for the treatment of the disease. A scientific base has been provided for these observations, wherein the hypoglycemic effect (of 50–200 mg/kg/day) of the seeds' aqueous extract was observed in normal, high glucose, and nicotine-induced hyperglycemic rats, which were possibly mediated via intestinal glucose uptake and adrenergic inhibition, respectively (Adeneye and Adeyemi, 2009a). Additionally, Adeneye and Adeyemi (2009b) reported hypoglycemic and antihyperlipidemic effects of the seed extracts that were mediated via enhanced peripheral glucose uptake and improvements in hyperinsulinemia.

7.5.3 *Microula sikkimensis* (Hemsl.)

Microula sikkimensis (Hemsl.) is a native oil plant species distributed in Bhutan, India, Nepal, and the northwestern region of China that is widely used as medicine, food, and for

fodder production, as well as water conservation (Wang et al., 2003). The seed oil has been reported to decrease cholesterol and triglyceride levels in blood serum, with an increase in the ratio between total cholesterol and high-density lipoprotein (Long and Ming, 2007). Seeds are also reported to prevent the accumulation of atheroma, to preserve the structural integrity of biological and inner membranes of vessels, and to alleviate high levels of blood fat (Li et al., 1999a,b). The lipids or the seed oil is reported to be an important source for treatment of cardiovascular and hepatic diseases (Liu et al., 2006). Additionally, the seeds are reported to be rich in unsaturated fatty acids (Cao and Suo, 2010).

7.5.4 Chinese chive seeds (*Allium tuberosum* Rottl.)

Chinese chive is one of the popular edible green vegetables of China and is used as both a food and a medicine. In Chinese folk medicine, the seeds are used as a tonic and aphrodisiac (Jiangsu New Medical College, 1986). In Thailand, the seeds are used for toothache, but not much scientific data is available on this**.** Chive seeds are composed of saponins, alkaloids, and amides (Sang et al., 1999, 2000a,b; Zou et al., 2001). The seed extract has been reported to show effective warming of the kidneys and to enhance the resistance of emasculated rats to cold and tiredness with increased autonomous activity (Wang et al., 2005).

7.5.5 Grape seeds (*Vitis vinifera* L.)

Grapes are one of the most popular fruits consumed worldwide due to their taste and wide variety of uses (in jams, juices, and wine), as well as for their high nutritional value. Grape seeds are reported to possess several therapeutic value. They are believed to provide health benefits mainly due to the presence of flavonoids in the form of proanthocyanidins (condensed tannins) that are present as procyanidins and prodelphinidins. The procyanidin obtained from grapeseed extract has been reported to possess *in vivo* antioxidant activity (Sato et al., 2001), is able to reduce lipid peroxidation (Bouhamidi et al., 1998), and can inhibit the production of free radicals (Bagchi et al., 1998). The other major compounds in seeds, apart from flavonoids (catechin, epicatechin, procyanidins, and anthocyanins), include phenolic acids (gallic acid and ellagic acid) and stilbenes (resveratrol and piceid). Flavonoids in grape seeds are reported to exhibit activities that work against peptic ulcers and several dermal disorders. The proanthocyanidins have been shown to have dermal wound healing properties (Khanna et al., 2002).

Recently, Choi and Lee (2009) evaluated antioxidant and antiproliferative activities of the tocotrienol-rich fraction (TRF) obtained from grape seeds and found that TRF, which is a mixture of c-tocopherol and a- and c-tocotrienol, exhibits significantly higher antioxidant and antiproliferative activities against breast and colon cancer cells. Saito et al. (1998) reported grape seed extracts with either high or low flavanol contents to show antiulcer activity in a rat model system. Grape seed extract containing oligomeric and polymeric proanthocyanidins has been also shown to possess antitumor activity in mouse epidermis (Bomser et al., 1999). A significant reduction in the free radicals including superoxide anions in mouse macrophages on administration of water–ethanol extracts of red grape seeds has been reported by Bagchi et al. (1998). From their study, they also found the seed extracts to reduce lipid peroxidation in liver and brain. *In vivo* studies have shown reduction in myocardial infarction rate with dietary supplementation of grape seed extract, and the supplementation of the seed extracts (1% w/w) to the diet showed a reduction in

atherosclerosis in the aorta without influencing the serum lipid profiles of rabbits (Sato et al., 1999; Yamakoshi et al., 1999). Joshi et al. (2000) have theorized that the seed extracts with high phenolic acid and gallic acid might play a significant role in inducing apoptosis in the body (Joshi et al., 2000). Additionally, according to Khanna et al. (2001), grape seeds containing 5 mg/g *trans*-resveratrol can be used to treat dermal wounds and other dermal disorders.

With multiple uses of grape seeds and their extracts, several patents have been awarded with regard to the processing and preparation of various commercial forms of grape seeds and related flavonoids as functional foods (Goodman, 2000, 2001; Carson et al., 2001; Ray and Bagchi, 2001).

7.5.6 Pumpkin seeds (*Cucurbita* sp.)

The pumpkin is one of the most popular seed vegetables and is widely popular in Asia, used for various culinary (from curry to sweet preparations) and medicinal purposes. Worldwide, there are about six species of pumpkin cultivated, among which *Cucurbita pepo* L., *Cucurbita maxima* Duchesne, and *Cucurbita moschata* Duchesne are the most economically important species (Taylor and Brant, 2002; Brent Loy, 2004). Pumpkin seeds are a good source of minerals, amino acids, β-carotene, and antioxidants (Akwaowo et al., 2000; Glew et al., 2006; Stevenson et al., 2007). The seed oil has been shown to have broad-spectrum antimicrobial activity. Wang and Ng (2003a) isolated a novel antifungal peptide (cucurmoschin) from pumpkin seeds that showed protection against *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella arachidicola*. Pumpkin seed oil is also known to help in modifying several altered parameters affected during arthritis, mainly in the chronic phase stage (Fahim et al., 1995). Pumpkin seed oil has also been shown to be effective against hypercholesterolemia (Zuhair et al., 1997). Treatment of spontaneously hypertensive rats with felodipine or captopril monotherapy or combined with pumpkin seed oil produced improvement in the measured free radical scavengers in the heart and kidney (Zuhair et al., 2000). Cai et al. (2003) have reported the hypoglycaemic action of pumpkin seed protein.

Reduction in the incidence of bladder stone by supplementation of pumpkin seeds for orthophosphate (60 mg/kg/day BW) has been reported by Suphakarn et al. (1987). Also, inhibition of crystal formation or aggregation leading to reduced risk of bladder stone disease has been reported on consuming pumpkin seed snacks (Suphiphat et al., 1993). Pumpkin seeds contain high amounts of fatty acids and phytosterols, which can be used for the treatment of benign prostatic hyperplasia. Seeds have also been used in traditional medicine as a vermifuge (Zhang et al., 1994; Dvorkin and Song, 2002). Seed proteins are reported to possess hypoglycemic action and can inhibit trypsin and activated Hageman factor (a serine protease involved in blood coagulation) (Krishnamoorthi et al., 1990; Dannenhoffer et al., 2001). In preclinical studies conducted by Diaz Obregon et al. (2004), an antihelminthic effect of pumpkin seeds has been reported. According to Huang et al. (2004), diets rich in pumpkin seeds could help in lowering the levels of gastric, breast, lung, and colorectal cancer. Makni et al. (2008) reported a mixture of flax and pumpkin seeds to have antiatherogenic and hepatoprotective effects, which were inferred to be mediated by unsaturated fatty acids present in the seed mixture. Recently, seed extracts of four different commercial pumpkin varieties have been shown to exhibit high antioxidant and lipoxygenase inhibitory activities (Xanthopoulou et al., 2009).

7.5.7 Horse chestnut seeds (*Aesculus hippocastanum* L.)

Horse chestnut is a medicinal plant that grows naturally in parts of Asia and in some parts of Europe and North America. Chestnut seeds have been shown to contain high amounts of triterpenoidal saponins called "aescins" that are known to promote increased blood circulation and that are useful for chronic venous insufficiency and varicose veins (Yoshikawa et al., 1994, 1996). The effectiveness of horse chestnut seed extract for treating chronic venous insufficiency has been reported by Pittler and Ernst (1998). Furthermore, seeds have been reported to possess anti-inflammatory, antiexudative, antiedematous, and capillaro-protective activities, as well as astringent properties (Vogel et al., 1970; Rothkopf and Vogel, 1976; Ody, 1993; Guillaume and Padioleau, 1994; Matsuda et al., 1997; Sirori, 2001). The antiobesity effects of novel saponins from edible seeds of Japanese horse chestnut have also been reported, wherein individual components of saponins inhibited pancreatic lipase *in vitro* (Kimura et al., 2008).

There is one more plant, commonly referred to as Chinese chestnut (*Castanea mollisima*), that has been reported to have medicinal value. Wang and Ng (2003b) isolated a novel antifungal protein-castamollin from the seeds that showed inhibitory activities against human immunodeficiency virus-1 reverse transcriptase (in a cell-free rabbit reticulocyte lysate system). Additionally, the authors reported that castamollin displays significant antifungal activity (against *Botrytis cinerea, Mycosphaerella arachidicola, Physalospora piricola*, and *Coprinus comatus*).

Apart from the seeds discussed in this chapter, there are also several other commonly encountered seeds with traditionally known medicinal benefits, namely, *Cassia absus*, *Datura fastusa, Ipomea bilaba, Jatropha curcas, Mimosa pudica, Nelumbium specialum, Rauwolfia serpentina, Ricinus communis, Vinca rosea,* and *Wrightia tinctoria*. However, the scientific background for the health benefits of these seeds still remains obscure or sources are scarce, and a gap needs to be filled in further exploration of their medicinal and therapeutic benefits.

7.6 FUTURE OUTLOOK

From the available database and scientific reports, it is a well-understood and accepted fact that plant seeds possess high medicinal and therapeutic value. However, as of now, most of the research on the subject has concentrated more on exploring popular or easily available seed resources rather than looking for underutilized seeds. Hence, as there is a wide gap in this, future studies exploring the medicinal benefits of underutilized or wild seeds are warranted**.**

Future studies must be pursued to identify and characterize various types of bioactive compounds present in the seeds as well as to study the efficiency of those compounds in curing particular ailments. With regard to the presence of toxic compounds and antinutrients in the seeds, various recently developed food processing treatments could be applied to overcome this. Additionally, physical methods of processing such as application of ionizing radiation, ultrasound, or ozone treatments can help in assuring the safety (microbial) as well as enhancing the bioactive compounds of these compounds. With the available worldwide infrastructure and increased knowledge and understanding of the various mechanisms involved in the development of an individual disease, it is envisaged that plant seeds might contribute significantly to solving many problems, at least up to a certain extent.

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8 Effects of Carotenoids and Retinoids on Immune-Mediated Chronic Inflammation in Inflammatory Bowel Disease

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8.1 INTRODUCTION

The inflammatory bowel diseases (IBDs) include a complex of chronic inflammatory diseases resulting from the abnormal regulation of mucosal immune responses. During persistent inflammatory phase, the production of proinflammatory cytokines and reactive oxygen species (ROS) are increased by the stimulation of signal cascades, which in turn contribute to the progression of immune responses. Consequently, the cycle of chronic proinflammatory processes is initiated which leads to an alteration in redox balance in the immune system. A failure to terminate proinflammatory mechanisms will lead to an autoimmune response. However, evidence from current nutrient supplementation studies suggests that fruit and vegetable components such as carotenoids can be used to ameliorate chronic inflammatory diseases and prevent inflammation-associated development of cancer.

Carotenoids are exogenous antioxidants that have the potential to reduce intracellular oxidative stress generated from neutrophil respiratory bursts during the chronic inflammation phase and show strong immune regulatory activities in both animal and human systems. Administration of carotenoids in conjunction with other natural components may regulate gene expression, thereby suppressing the inflammatory responses. Accordingly, carotenoids contribute to the regulation of redox cell-signaling in the immune system and maintain a dynamic equilibrium of immune responses. A thorough understanding of carotenoid-chemopreventive activities will enable the development of effective carotenoid-based intervention strategies for patients suffering from IBD with high risk for developing colon cancer.

8.2 CAROTENOIDS

Carotenoids are photosynthetic plant pigments with high nutritional value and are represented by more than 600 known natural structural variants. For instance, beta-carotene is

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an important carotenoid responsible for regulation of harvesting light energy during photosynthesis, and helps stabilize other pigments and chloroplasts from excessive free radical damage. Beta-carotene is the major storage compound in carrots. In addition to beta-carotene, a precursor of carotene, for example, lycopene, is an important red pigment in fruits such as tomato and melon and is itself associated with several health benefits. Xanthophylls are hydroxylated derivatives of beta-carotene. Beta-carotene is also a main precursor of vitamin A in mammals. Dietary carotenoids are synthesized through the isoprenoid pathway from geranylgeranyl pyrophosphate (GGPP) through several metabolic steps, and are present as alpha- and beta-carotenes in plants. Of these dietary carotenoids, beta-carotene, referred to as pro-vitamin A, has various biological functions in vertebrates. The biosynthesis of vitamin A is a process whereby beta-carotene is converted into vitamin A in the animal body after being absorbed by the small intestine. Because of its particular structural and biological characteristics, beta-carotene can contribute to the enhancement of human and animal health. Beta-carotene, as well as other kinds of carotenoids, has extended conjugated double bonds that are capable of quenching ROS. Thus, the carotenoids have been recognized as important dietary antioxidants in recent decades. Dietary carotenoids can provide abundant health benefits, including cancer prevention, immune modulation, and anti-inflammatory capability. Therefore, there is an increasing interest in using carotenoids for disease prevention through dietary intervention.

8.3 IBDS

IBD, a group of immune-mediated intestinal inflammatory diseases induced by environmental stimulation and genetic susceptibility (Cho, 2006), is a typical inflammatory immune disease. IBD includes two main types of diseases: Crohn's disease (CD) and ulcerative colitis (UC). Currently, there is no comprehensive understanding of the etiology and mechanisms of IBD. The possible pathogenic process of IBD in humans is that the persistent infections caused by environmental influences, commensal enteric bacteria, or antigens from foods initiate acute and chronic intestinal inflammation. Consequently, this inflammation results in destruction of mucosal barriers and a dysregulation of the mucosal immune system. Due to migration of populations and environmental changes, the prevalence of IBD has increased in the past three to four decades (Cho, 2006). Currently, about 1.4 million people suffer from IBD in United States (CDC, 2007). No significant population-specific differences in the prevalence of IBD have been identified among Canadians (CDC, 2007). Previously, the prevalence rate of IBD was believed to be high in North America and Northern Europe. However, more recently, its prevalence rate has begun to rise in low-incidence areas such as Southern Europe and Asia. Therefore, the need to develop effective strategies to prevent the development of IBD is urgent. There are currently no identified preventive strategies or effective treatments for IBD. The immunogenic mechanisms leading to IBD are complex, because the two types of IBD show different immune responses to intestinal infections. Also, there is an increased risk for colon cancer development as a result of prolonged IBD. However, the mechanisms leading to colon cancer development in IBD patients are unclear.

Intestinal mucosal immunity is critical for human and animal health because it is the first line of defense against pathogen invasion and infection. An abnormal intestinal mucosal immune system can develop in patients who suffer from IBD, and this can result in an inflammatory autoimmune response. Even though numerous novel therapeutic approaches are currently being developed for IBD, these approaches are based on suppressing the

immune responses by the use of drugs (steroids and nonsteroidal anti-inflammatory drugs) or through using specific antibodies to block proinflammatory cytokines. However, excessive immune suppression may increase the risk of developing cancer (Baniyash, 2006). Thus, immune suppression therapy may not necessarily have an enhancing effect on patients' health in the long term.

8.4 PHYTOCHEMICALS AND DOWNREGULATION OF IBD

Nutritional and dietary interventions have recently become potential complementary strategies for downregulating various inflammatory diseases. The essential nutrients have been shown to play a regulatory role in human and animal immune systems (Willcox et al., 2004). Recent research has shown that the patients with CD in Canada required micronutrient supplementation (Aghdassi et al., 2007). Based on these findings, several vitamins and phytochemicals are being used for reducing symptoms of IBD. Among these phytochemicals, carotenoids are the outstanding candidates for amelioration of IBD because of their potency as antioxidants and regulators of inflammatory immune responses. The main biofunctions of carotenoids are antioxidant activity, immune modulation, and regulation of gene expression. The following sections will focus on these three aspects of beta-carotene to determine its potential role in amelioration of IBD.

8.4.1 Antioxidative capacity of carotenoids to reduce oxidative stress generated from inflammation

The condition of increasing oxidative stress with decreasing antioxidant defenses has been identified in colonic mucosal biopsies of patients with IBD (Lih-Brody et al., 1996). This condition of oxidative stress is generated from oxidative respiratory bursts during the phase of chronic intestinal inflammation. The oxidative respiratory burst is a characteristic mechanism of the immune response to eliminate bacteria and is induced by immune cells, including phagocytes, neutrophils, and macrophages. In the majority of animal species, the endogenous antioxidative system protects them from the free radical-induced oxidative damage, because the system is composed of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR). However, the persisting endogenous oxidative stress generated during chronic intestinal inflammation phase overwhelms the normal endogenous antioxidants and results in the destruction of the antioxidant defense system (Vandana et al., 2006). Thus, exogenous antioxidants from diet, such as vitamins E and C and carotenoids (beta-carotene, lycopene, xanthophylls such as lutein) are optimal candidates for enhancing the function of the antioxidant defense system during the inflammation phase. Carotenoids have been confirmed to have a beneficial effect on the immune system *in vivo* (McDevitt et al., 2005). In addition, Kawakami et al. (2007) identified a significant correlation between the serum oxygen radical scavenging capacity and beta-carotene and retinol concentrations in UC patients. Consequently, carotenoid supplementation is a potential nutritional intervention for patients suffering from IBD.

Carotenoids can effectively quench ROS generated by exogenous and endogenous factors, thereby protecting cellular DNA, membrane lipids, and proteins from oxidative damage. Bohne et al. (1997) showed that the antioxidant action of beta-carotene reduced the level of ROS generated by neutrophils under ultraviolet light (UV) radiation. Current research has validated that lycopene possessed better chemopreventive activity than beta-carotene in mitigating oxidative damage in tissue under UV exposure, but both chemicals

contributed to the reduction in lipoperoxide levels (Adhami et al., 2008). Both beta-carotene and lycopene have been shown to protect low-density lipoprotein (LDL) from oxidization (Jialal and Grundy, 1992; Rao and Agarwal, 1999). LDL can be oxidized *in vivo* by myeloperoxidase (MPO), an intracellular enzyme secreted by macrophages and neutrophils, thereby aggravating inflammation (Itabe, 2003). MPO can act as an indicator of neutrophil infiltration at sites of the damaged colon. Tran et al. (2007) detected increasing activity of MPO in experimental IBD. Even though a direct identification of oxidized-LDL (ox-LDL) in IBD has not been reported, CXCL16 (chemokine (C-X-C motif) ligand 16), a small cytokine induced by the interferons (IFN)-γ and tumor necrosis factor (TNF)-α, a transmembrane protein functioning as a scavenger receptor for ox-LDL, has been recently identified in the blood of both CD and UC patients (Lehrke et al., 2008). Accordingly, carotenoids may reduce the levels of ox-LDL in IBD by influencing the expression of CXCL16. Thus, carotenoid intervention can potentially reduce ROS secreted by granulocytes.

An increase in ROS has been identified in both UC and CD (Pavlick et al., 2002; Seril et al., 2006). ROS contribute to redox imbalance of inflammatory autoimmune disease and induce the intestinal epithelial lesions. In addition, they play critical roles in modulation of the inflammatory response in IBD (Pavlick et al., 2002). In IBD, the production of ROS and nitric oxide (NO) occurs through different pathways. First, the activation of macrophages and phagocytic leukocytes (granulocytes, eosinophils, and monocytes) results in the generation of a large amount of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) induced by NADPH oxidase. Activation of macrophages and granulocytes can enhance the production of hypochlorous acid (HOCl) via MPO in IBD. Also, upregulation of inducible nitric oxide synthase (iNOS) in IBD results in overproduction of NO, which plays a key role in anti- and proinflammatory mechanisms (Pavlick et al., 2002). As demonstrated in current studies (Kennedy and Liebler, 1992; Mortensen et al., 1997; Sommerburg et al., 2003), the dietary carotenoids can scavenge intracellular ROS at different steps of the pathway, resulting in the downregulation of activated oxygen species, and thus may play a key role in antioxidant defense mechanisms against oxidative stress and in keeping redox homeostasis in IBD (Fig. 8.1).

However, the intracellular antioxidant capacity of beta-carotene has recently been questioned, because the cleavage products (CPs) of beta-carotene show more genotoxic effects on cells under oxidative stress (Siems et al., 2003; Alijia et al., 2006). Accordingly, supplementation with high concentrations of beta-carotene can increase the cellular oxidative damage when cells are exposed to serious oxidative stress. The degradation of beta-carotene leads to the production of epoxides at the beta-ionone ring and aldehydes with different chain lengths (Sies et al., 1992); these CPs are highly reactive and are potentially toxic to cells. Thus, other kinds of carotenoids may be more promising as exogenous antioxidants, particularly lycopene. In addition, the capacity of lycopene to quench radicals is more extensive than beta-carotene as indicated in a recent study (Miller et al., 1996). Accordingly, lycopene, referred to as an optimal exogenous antioxidant, has more potential to ameliorate inflammatory diseases (Rao and Agarwal, 1999; Tapiero et al., 2004; Jacob et al., 2008). However, moderate quantitative dietary beta-carotene may still be beneficial for restoring cellular redox homeostasis and reduction of inflammation.

8.4.2 Immune-modulating activity of carotenoids

CD and UC cause excessive immune responses and persistent inflammation in the intestinal epithelia and gut-associated lymphoid tissue (GALT). The main objective of regulating

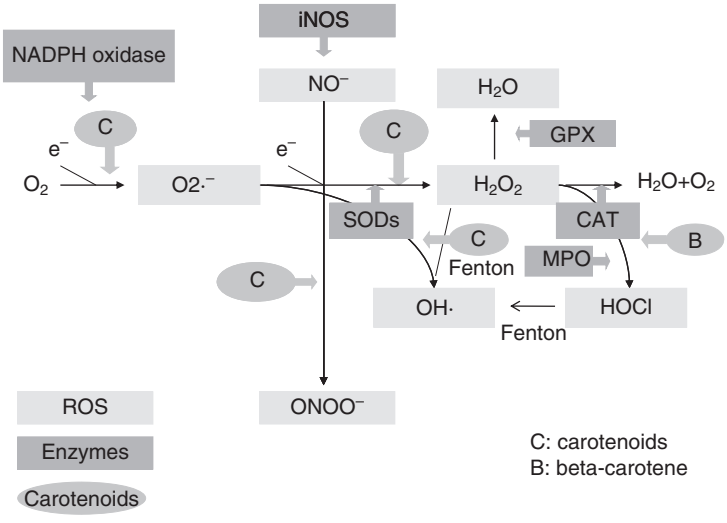


Figure 8.1 Represents the progress of reactive oxygen species (ROS) production and carotenoids involved in antioxidant mechanism. The endogenous antioxidant enzymes (SODs, catalase, and GPx) can remove most of peroxyl radicals and superoxide anion. Beta-carotene as well as other carotenoids effectively scavenge free radicals and reduce the oxidative stress caused by peroxyl radicals, nitrogen dioxide, and hypochlorous acid. Therefore, carotenoids can contribute to the restoration of cellular redox homeostasis.

inflammatory immune responses in IBD is to restore the homeostasis in the mucosal immune system and the phagocytosis mediated by leukocytes.

8.4.2.1 Modulating proliferation of macrophages and granulocytes

Phagocytes are white blood cells that are involved in immunoprotection by scavenging foreign particles such as invading pathogens. There are two main types of phagocytes: monocytes and polymorphonuclear leukocytes (PMNs). PMNs, also referred to as granulocytes, include neutrophils, eosinophils, and basophils. The neutrophil is a typical granulocyte and plays a critical role in the intestinal defense system. After being stimulated by bacterial infection, neutrophils can migrate to the site of inflammation from the vasculature and release bactericidal compounds that are mainly ROS. This is called the degranulation process of neutrophils and is also referred to as a neutrophil respiratory burst. The degranulation of neutrophils is characteristic of an acute inflammation. Intestinal macrophages, on the other hand, act primarily as phagocytic cells to remove the dead cell debris of pathogens and neutrophils. Macrophages, which release a variety of proinflammatory cytokines to activate lymphocytic responses, play a vital role in acute and chronic inflammation of IBD (Leon et al., 2006). Furthermore, the chronic inflammation can increase the production of tumor-associated macrophages (TAMs). TAMs mainly promote tumor proliferation and progression, as well as inhibit adaptive immunity (Coussens and Werb, 2002; Balkwill et al., 2005). Accordingly, the overreactive intestinal macrophages in a chronic inflammation may induce an increased risk of developing colon cancer for patients suffering from IBD.

The immune regulating activity of carotenoids has been identified in several studies. It has been demonstrated that carotenoids have regulating effects on innate immune responses

including the activity of the macrophages and neutrophils (Terasa et al., 2005; Walrand et al., 2005). Currently, *in vivo* and *in vitro* evidences indicate that administration of beta-carotene can reduce the oxidative stress in macrophages and neutrophils (Imamura et al., 2006). Imamura et al. (2006) showed that beta-carotene can modulate cytoplasmic redox status of the macrophage by enhancing the level of intracellular glutathione, which is an endogenous antioxidant. Walrand et al. (2005) provided *in vivo* and *in vitro* evidences to establish that carotenoids can modulate PMN oxidative bursts at physiological levels and may prevent collateral tissue damage during inflammation. However, it should be noted that carotenoid CPs might cause negative effects on PMNs. Siems et al. (2003) and Alijia et al. (2006) showed that a higher concentration of beta-carotene CPs have a proapoptotic effect on human neutrophils *in vitro*. The controversial physiological role of beta-carotene identified in the previous studies is important for an in-depth understanding of the antioxidant capacity of beta-carotene. A comprehensive understanding of the function of carotenoid CPs still needs to be addressed in further studies.

The other type of phagocytes, macrophages, is also regulated by carotenoids and their degradative product retinoid (Stephensen, 2001; Terasa et al., 2005; Ruhl, 2007; Rafi et al., 2007). Terasa et al. (2005) established that the carotenoids could inhibit macrophage proliferation in chronic diseases. Beta-carotene also shows a moderately suppressive effect on murine macrophage proliferation. Subsequently, Rafi et al. (2007) verified that lycopene has anti-inflammatory activity that can reduce the mRNA expression and production of iNOS at the protein level in murine macrophages. Lycopene can also potentially promote macrophage differentiation (Terasa et al., 2005).

The retinoids and retinoic acid (RA) produced from beta-carotene also have effects on macrophage activation because vitamin A deficiency leads to an increase in macrophage-mediated inflammation and production of interleukin (IL)-12 and Interferons (IFN)-γ (Stephensen, 2001; Ruhl, 2007). Vitamin A supplementation can reduce inflammation by diminishing the level of proinflammatory cytokines such as tumor necrosis factors (TNF)-α, and enhance phagocytic activity of macrophages against the pathogen invasion (Stephensen, 2001).

8.4.2.2 Mechanisms regulating lymphocytic immune responses by carotenoids

Various immune mechanisms involved in the pathogenesis of IBD (Radford-Smith and Pandeya, 2006; Sartor, 2006) include several components. The proinflammatory molecules, such as TNF-α and IFN-γ which can upregulate CARD15/NOD2 [(nucleotide-binding oligomerization domain containing 2, which is also known as the caspase recruitment domain family member 15 (CARD15)] play an important role in the regulation of immune system function. The NOD2 gene is linked to inflammatory diseases such as IBD and UC (Radford-Smith and Pandeya, 2006). NOD2 is also involved in bacterial recognition and the perpetuation of the inflammation in CD. The recruitment of macrophages is triggered by the upregulated expression of recognition receptors and enhanced by proinflammatory cytokines. In IBD, the recruitment of macrophages is persistent and results in the stimulation of the secretion of abundant proinflammatory cytokines to increase the severity of inflammation. At the same time, the lipopolysaccharides (LPSs) on bacterial surfaces stimulate the expression of intercellular adhesion molecule (ICAM), cyclooxygenase 2 (COX-2), and IL-6 in intestinal epithelial cells, and activate nuclear factor-kappa B (NF-κB) pathway which enhances inflammation. The LPS can also activate naïve dendritic cells (DCs) and stimulate the secretion of IL-12 and expression of the major histocompatibility

complex (MHC) II molecules, which is critical for the initiation and regulation of adaptive immune responses in the mucosal immune system (Friese et al., 2005). The T-helper 1 (Th1) responses are channeled through this pathway. Eventually, the failure of antigen elimination in chronic IBD and the persistence of various nonspecific biological amplification mechanisms contribute to the development of inordinate humoral immune responses, resulting in an autoimmune disease. This situation is more significant in UC patients. The suspicious autoantigen and autoantibody responses are mainly identified in UC patients because the excessively stimulated T-helper 2 (Th2) responses which are essential to induce humoral immune responses cause the disruption of the intestinal epithelium (Brandtzaeg et al., 2006). Of these mechanisms, the influences of the carotenoids on phagocytosis have been summarized in the previous section. The potential regulating role of carotenoids on lymphocytic immune responses is discussed in the following sections.

The dysregulation of mucosal immunity in IBD is primarily caused by a persistent cluster of differentiation 4 ($CD4^+$) T lymphocyte cell-mediated immune responses mainly including Th1 cell, Th2 cell, and regulatory T cell (Treg) responses. Activation of both Th1 and Th2 responses are significantly increased in IBD. Maintaining the balance between Th1 and Th2 is less critical in IBD compared with other types of autoimmune diseases such as type I autoimmune diabetes, and food allergies which cause intestinal immune disorders. Current evidences indicate that both plant-derived carotenoids and biosynthesized retinoids may play a key role in regulation of immune systems in various animal species, such as avian, canine, and felid (Chew et al., 2000; Kim et al., 2000; Horak et al., 2006). In particular, dietary carotenoids are integrated with ecophysiological functions in avian species. This suggests that the carotenoids may be micronutrients that may regulate immune function to protect hosts against specific pathogen infections (Long and Nanthakumar, 2004). Thus, the immune regulating activities of carotenoids and retinods may be based on their chemostructural properties and the pathogenic specificity in the host. Recently, it has been demonstrated that higher concentrations of dietary beta-carotene and vitamin A shows immune-enhancive effects on host immune systems by affecting the differentiation and expansion of immune cells (Garcia et al., 2003; Ruhl, 2007).

A proinflammatory Th1 response dominates in Crohn's disease, which is stimulated by large amounts of IFN-γ and IL-12 (Head and Jurenka, 2003; Elson et al., 2006). The proinflammator IL-12 produced by macrophages plays a central role in T cell differentiation and triggers the development of Th1 responses. Carotenoids and retinoids can modulate the production of proinflammatory cytokines and regulate Th1/Th2 balance. Beta-carotene supplementation contributes to the downregulation of the expression of IL-12 in a murine macrophage cell line (Imamura et al., 2006). As well, lycopene has been shown to inhibit LPS-induced IL-12 produced by murine DC (Kim et al., 2004). Vitamin A supplementation can inhibit the secretion of IL-12 and IFN-γ (Stephensen, 2001; Haog et al., 2002; Ruhl, 2007). The retinoids including vitamin A and RA can shift the Th1 response to the Th2 response by increasing IL-4 as demonstrated in several studies (Stephensen, 2001; Long et al., 2006; Ruhl, 2007). In CD, the shift in the immune response from Th1 to Th2 can ameliorate the mucosal inflammation induced by overreactive Th1 cells and promote the development of an intestinal humoral immune response (HIR) against specific pathogenic bacteria invasion. In a recent study, Long et al. (2006) identified the different modulating effects of vitamin A supplementation on levels of IL-4 and IFN-γ in response to pathogen induced diarrhea, and these effects depend on the specific type of enteric pathogen infection. Nevertheless, some retinoids may directly suppress the Th1 response. Massacesi et al. (1991) determined that there was a suppressive effect of 13-cis-retinoic acid on IL-2

activity and Th1 response in rats. These results show that 13-cis-retinoic acid may also potentially contribute to the suppression of Th1-mediated autoimmune disease.

Current evidences demonstrate that the UC might be dominated by Th2-mediated humoral immune response (Head and Jurenka, 2003; Elson et al., 2006). In UC, the colonic inflammation is mediated by an excessive Th2 response, stimulated by an increase of Th2-specific cytokines secretion including IL-5, IL-4, and IL-13 (Fuss et al., 1996; Elson et al., 2006). Carotenoids and retinoids also show regulating effects on Th2 response. Vitamin A supplementation can enhance IL-4, IL-5, and IL-10 cytokine secretion (Stephensen, 2001; Long and Nanthakumar, 2004; Ruhl, 2007). The direct modulating effects of beta-carotene on the production of IL-4 and IL-5 are not defined. Lycopene and lutein did not influence the production of IL-5 (Iyonouchi et al., 1996). However, Koizumi et al. (2006) indicated that beta-carotene and supplemental α-tocopherol promoted IL-12 secretion and enhanced the Th2 cell proliferation in DO11.10 mice splenocytes after being treated with ovalbumin (OVA) antigen. Thus, the effects of carotenoids on inflammatory immune responses may depend on the specific antigen by which the dominant T-cell response is stimulated. Furthermore, the nutrient intervention studies indicated that dietary vegetable juice, which contains a lower concentration of carotenoids, has a more positive effect on modulation of human T lymphocyte functions (Watzl et al., 1999; Briviba et al., 2004). Watzl et al. (1999) identified that the secretion of human IL-2 (Th1 cells) and IL-4 (Th2 cells) were suppressed by consumption of low-carotenoid vegetable juice, and T lymphocyte function could be restored by consumption of tomato juice which contains a higher level of lycopene. Moreover, in their continuing study, Briviba et al. (2004) detected that the consumption of tomato juice suppressed IL-4 overproduction in smokers. However, the molecular mechanisms involved in carotenoids and retinoids regulating expression of Th2-specific cytokines are still unclear and need to be elucidated by further studies.

In addition to the influences of carotenoids and retinoids on T-cell responses, they also have regulating effects on immunoglobulin secretion in B lymphocytes (Jyonouchi et al., 1994; Stephensen, 2001; Long and Nanthakumar, 2004; Ruhl, 2007). The level of immunoglobulin (Ig) A is very low in IBD. IgA is important to protect against mucosal infection, and maintain mucosal immune homeostasis in IBD (Brandtzaeg et al., 2006). High levels of dietary vitamin A significantly enhanced the IgA response and IL-10 production, and reduced the IgG response in mice with respiratory tract infection (Cui et al., 2000). This indicates that vitamin A supplements may contribute to regulating IgA-mediated responses to extracellular infection and restoring the homeostasis of the mucosal immunity. Nikawa et al. (1999) also showed that vitamin A prevented the decline in IgA in small intestinal mucosa. The carotenoid supplementation including that of lutein, astaxanthin, and beta-carotene resulted in increased antibody production such as that of IgM and IgG in older animals (Jyonouchi et al., 1994). Furthermore, localized antibodies of the IgG1 subclass spontaneously produced by mononuclear cells from the active UC lesions are suspicious autoantibodies, which occur in the colon epithelial protein (CEP) fraction (Brandtzaeg et al., 2006). Tokuyama and Tokuyama (1996) showed that the retinoids are crucial to modulate the initiation and direction of the shift in isotype profiles of IgA and IgG1. They also determined that RA enhanced IgA production in the presence of IL-5. However, the specific modulating effects of carotenoid and retinoid supplementation on UC are not defined. Further studies are required to determine the influences of carotenoid or retinoid interventions on Th2-mediated responses in UC.

The last type of T-lymphocytic response currently identified in the IBD is the Treg response which plays a central role in chronic inflammatory diseases. Treg lymphocytes,

also called suppressor T cells, can inhibit persistent Th1 and Th2 immune responses and induce tolerance to persistent infection, thereby maintaining the homeostasis of the immune system (Long and Nanthakumar, 2004; Driel and Ang, 2008). Present evidences suggest that the pathogenesis of IBD is caused by the failure of activating Treg response (MacDonald and Monteleone, 2006; Werner and Haller, 2007; Driel and Ang, 2008). Current studies also show that chronic intestinal inflammation can be developed in animals depleted of IL-10, or transforming growth factor beta (TGF-β), which are secreted by Treg cells to regulate immune responses(Laroux et al., 2001). Treg cells can restore the balance of the antigen-driven naïve memory T-cell responses and inhibit the development of intestinal epithelial injury by producing regulatory cytokines IL-10 and TGF-β. T-helper 3 (Th3) cells are developed from Treg cells and have the function of protecting the intestinal mucosal epithelial system. Their response can reduce the formation of macrophage-derived reactive oxygen metabolites (ROM) and stimulate the production of TGF-β (Laroux et al., 2001). Carotenoids and retinoids have shown an influence on the development of Treg response. Sun et al. (2007) demonstrated that optimal production of Foxp3 Treg cells in GALT was dependent on the cooperation between TGF-β and RA. Coombes et al. (2007) also identified that RA might be a cofactor in the development of Treg responses induced by DCs in GALT. In addition, Cui et al. (2000) indicated that the level of IL-10 was significantly increased in infected mice treated with dietary vitamin A. However, there is limited information available from current studies about the enhancing effects of carotenoids, such as beta-carotene, lutein, and lycopene on production of TGF-β and IL-10. Gunasekera et al. (2007) showed that lycopene had no significant effect on TGF-β. Thus, the regulatory effects of carotenoid interventions on Treg response in IBD require further investigation.

Thus, the lymphocyte immune responses play a key role in the pathogenesis and physiology of the complex of chronic inflammatory diseases such as IBD. The multiple components of the immune system contribute to protecting the gut from a wide range of infectious agents and maintaining a dynamic equilibrium through their regulatory mechanisms. Immune responses of IBD involve components such as T-helper cell, including Th1, Th2, and Treg, that have different physiological roles in the development of chronic inflammatory immune diseases. The carotenoid and retinoid intervention is a potential therapeutic approach to adjust the dysregulated immune responses in IBD and reestablish the homeostasis of mucosal immune system as shown in Figure 8.2.

8.5 EFFECTS OF CAROTENOIDS ON IMMUNE GENETIC MECHANISM OF IBD

As mentioned previously, IBD is an inflammatory autoimmune disease (Baniyash, 2006; Elson et al., 2006). Inflammatory autoimmune diseases can be initiated by antigen-independent activation of the innate immune system under sterile conditions, which suggests the absence of pathogenic infection (Ohshima et al., 2005; Baniyash, 2006). Several types of cells involved in the innate immune system include the granulocytes, DCs, macrophages, natural killer (NK) cells and mast cells. Toll-like receptors (TLRs), located on the surface of the innate immune cells, recognize exogenous inducers which are antigens produced by enteric bacteria. The self-perpetuating inflammatory responses in IBD are stimulated by the direct or indirect activation of TLRs expressed on myeloid cells which result in a cascade of intracellular signaling events. Eventually, this signaling cascade leads

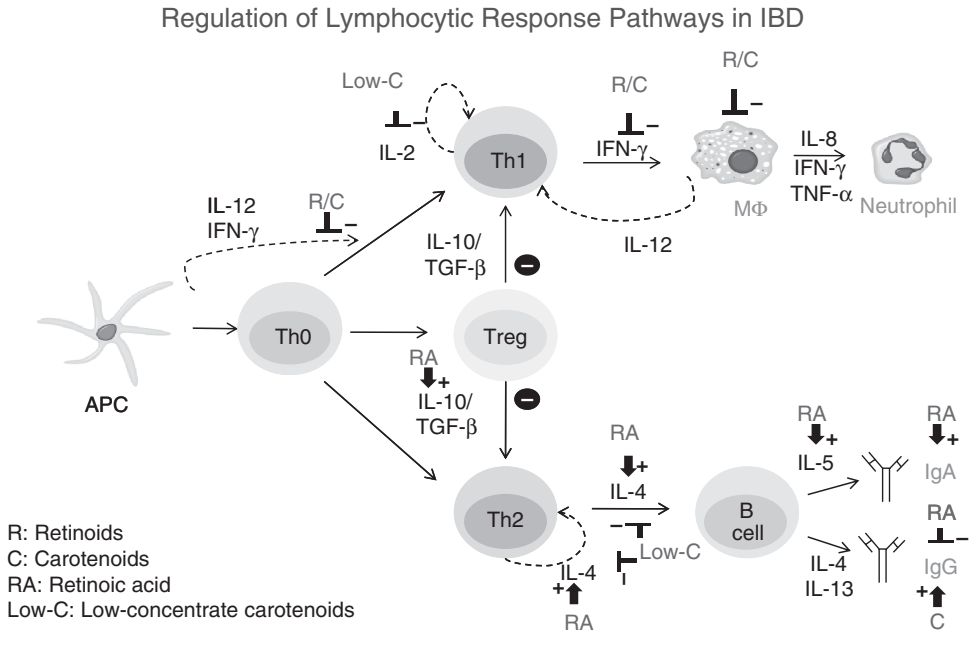


Figure 8.2 Schematic representation of the lymphocytic response pathways in IBD regulated by carotenoids and retinoids. After stimulation of antigen presenting cells (APC), the signal cascade is activated, and proinflammatory cytokines are produced. The T-helper (Th) 1 and Th2 responses are enhanced during the phase of chronic inflammation. Carotenoids and retinoids can modulate the Th1 and Th2 responses in the progression of the IBD through regulating proinflammatory cytokines and affecting proliferation of monocytes. MΦ: Macrophage +, ⬆: promoted; –, ⊥: inhibited; ⊖: suppressed.

to the activation of transcription factors which can induce and promote proinflammatory responses such as NF-κB and activator protein (AP-1). The novel therapeutic strategies used to mitigate the inflammatory responses in IBD are dependent on in-depth understanding of signal transduction associated with pathogenetic mechanisms of the disease. As an essential group of nutrient, carotenoids with provitamin A potentially have regulatory effects on inflammatory response due to their interaction with proinflammatory signaling pathways.

8.5.1 Potential role of retinoid receptors in attenuation of inflammatory diseases

Retinoids derived from dietary carotenoids play a critical role in the functional regulation of immune systems through directly influencing the gene transcription (Kuenzli et al., 2004; Spilianakis et al., 2005). Retinoids influence gene expression by binding to retinoid receptor complex including retinoic acid receptors (RARs), and retinoid X receptors (RXRs), which are also steroid hormone receptors. Dimers of RAR/RXR are localized in the nucleus, and bind with the retinoid-responsive DNA sequences through their DNA-binding protein hormone response elements (HREs). Accordingly, retinoids and carotenoids can regulate cellular gene expressions through HRE of retinoid receptor complex, which contains DNA recognition elements. For the retinoid receptor complex, the RAR can be activated by all-

trans retinoic acid (atRA), but the RXR is primarily activated by 9-cis RA (9cRA). Dietary carotenoids and retinoids potentially modulate the mucosal inflammation via the activation of the retinoid receptor complex, by which the defensive function of mucosal epithelial barriers might be enhanced (Kuenzli et al., 2004). Several studies have shown that retinoid receptors are directly and indirectly involved in cellular anti-inflammatory mechanism via their trans-repression and trans-activation capabilities (Caelles et al., 1997; Kuenzli et al., 2004; Altucci et al., 2007). The RAR-dependent signaling pathway may interact with the AP-1 and NF-κB-involved proinflammatory signaling transduction at a cellular level. Thus, the retinoid receptor complex may play a key role in regulating proinflammatory immune responses.

The activation of retinoid receptors shows inhibitory effects on expression of the transcription factor NF-κB. Na et al. (1999) indicated that NF-κB might constitutively interact with RXR, suggesting a negative cross-talk between the NF-κB and RXR complexes. The inhibition of NF-κB–DNA interaction and competitive recruitment of transcription integrators between NF-κB and RXR resulted after the administration of retinoids to macrophages (Na et al., 1999). Geissmann et al. (2003) showed that retinoids contribute to regulating apoptosis and maturation of DCs via the RXR/RAR pathway and influencing antigen-specific T-cell response. Yonezawa et al. (2007) indicated that the RAR-dependent signaling pathway was involved in the expression of nuclear factor of activated T cells (NFATs) c1 induced by NF-κB ligand (receptor activator of nuclear factor-kappa B ligand [RANKL]) in mouse monocytes. The activation of RANKL can be inhibited in monocytes after treatment with RAR agonists; therefore, RANKL-induced AP-1 and NFAT activations are suppressed. Current evidences indicate that retinoids can activate RAR/RXR heterodimers via their selective ligands, and then induce the inhibition of AP-1 action through trans-repressing AP-1 motifs (Thacher et al., 2000; Kuenzli et al., 2004; Papoutsaki et al., 2004). Disepio et al. (1997) and Chen et al. (1995) showed that RAR activated by retinoids could repress AP-1 and inhibit expression of IL-6 which plays an important role in inducing inflammation. Moreover, Kuenzli et al. (2004) indicated that RA might contribute to reducing matrix metalloproteinase (MMP) expression via activating RAR. Therefore, the activation of RAR/RXR heterodimer has the potent anti-inflammatory capability via trans-repression of inflammatory genes which can stimulate stress-signaling pathways.

Activation of RAR-dependent signaling pathway contributes to recruitment of co-activator or co-repressor proteins which are directly involved in regulation of T-cell responses (Stephensen et al., 2002). Iwata et al. (2004) determined that the binding of RA to RAR in T cells enhanced the expression of the gut-homing receptor, thereby recruiting T cells to the gut. In addition, the activation of RXR heterodimers has modulating effects on T-cell differentiation and Th1/Th2 balance by the involvement of transcription processes. Stephensen et al. (2002) indicated that RXR agonist enhanced the expression of IL-4 and transcription factor GATA binding protein 3 which plays a key role in Th2 differentiation. Thus, the RXR signaling pathway may play a critical role in regulating T-cell-mediated immune responses.

8.5.2 Modulation of inflammatory responses through activation of nuclear receptors containing RXR heterodimers

RXR can form heterodimers with various nuclear receptors such as vitamin D3 receptor (VDR) and peroxisome proliferator-activated receptors (PPARs). The activity of these receptors can directly interfere with proinflammatory pathways and suppress inflammation

Table 8.1. Potential anti-inflammatory effects of nuclear receptors on the production of various cytokines from immune cells

	RAR	RXR	PPARα	PPARβ/δ	PPARγ
Innate immune system	RANKL↓ AP-1↓ NFAT↓ IL-6↓ MMP↓		COX-2↓ IL-6↓		IL-8↓ IL-6↓ IL-1β↓ IL-12↓ iNOS↓ TNFα↓ MMP-9↓
Adaptative immune system Th1			IL-2↓ INFγ↓ TNFα↓		IL-2↓ INFγ↓ TNFα↓
Adaptative immune system Th2		IL-4↑ GATA3↑	IL-4↑		IL-4↑ IL-10↑ GATA3↑
Other cellular response			VCAM-1↓	Intestinal crypts↑	

(Stephensen et al., 2002; Kuenzli et al., 2004). Therefore, RXR is a crucial co-activator for the control of inflammation. The most important heterodimers involved in anti-inflammatory mechanism in IBD might be the RXR/PPARs. RXR is the main component in the RXR/PPAR complex. PPARs also play a key role in the intersection of multiple pathways involved in lipid metabolism and inflammation. PPARs have been identified to play a crucial role in regulating inflammatory pathways as a transcriptional regulator (Ahmed et al., 2007). The activation of PPARs and the resulting anti-inflammatory mechanism is determined by the transcriptional protein complexes formed and their phosphorylation. The expression of PPARs contributes to the repression of inflammatory gene transcription, and interferes with other transcription factors such as NF-κB, signal transducer and activator of transcription (STAT), AP-1, and NFAT. Thus, the activation of PPARs' ligands can modulate the onset of chronic inflammation and reduce autoimmune diseases (Genolet et al., 2004; Ahmed et al., 2007). PPARs primarily have four isotypes; PPARα, PPARβ, PPARγ, and PPARδ. They all have been shown to possess optimal modulatory effects on inflammation, which is summarized in Table 8.1 (Cabrero et al., 2002; Duez et al., 2006).

The interactions of PPARα with NF-κB and AP-1 transcriptional pathway to inhibit inflammatory responses are shown in Figure 8.3. The expression of PPARα might repress Th1-type cytokine production and favor the Th2 response (Genolet et al., 2004). In addition, the activation of PPARα has effects on cytokine-induced expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), thereby inhibiting leukocyte recruitment (Genolet et al., 2004). PPARα also has inhibitory effects on production of prostaglandins as well as the expression of COX-2 and IL-6(Genolet et al., 2004). The proinflammatory cytokine-induced PPARβ expression can enhance the healing program involving keratinocyte survival, proliferation, and differentiation (Tan et al., 2001). It has also been identified that PPARβ/δ contributed to the homeostasis of Paneth cell differentiation, which is a type of precursor cells in the small intestinal crypts (Varnat et al., 2006). The development of crypt basis is important to establish the intestinal defense system against pathogenic invasion. As well, PPARγ shows anti-inflammatory capability in IBD (Cabrero et al., 2002). The activation of PPARγ-specific ligands inhibits the expression of macrophage-produced proinflammatory proteins such as TNF-α, IL-1β, and IL-6, and the expression of iNOS and MMP-9 (Duez et al., 2006). The reason for this is that the expression of PPARγ can block the proinflammatory DNA binding activity of transcription factors through interacting with c-Jun, P65, P50, and NFAT (Cabrero et al., 2002). PPARγ plays a vital role in maintaining the balance of T-lymphocytic responses (Genolet et al., 2004).

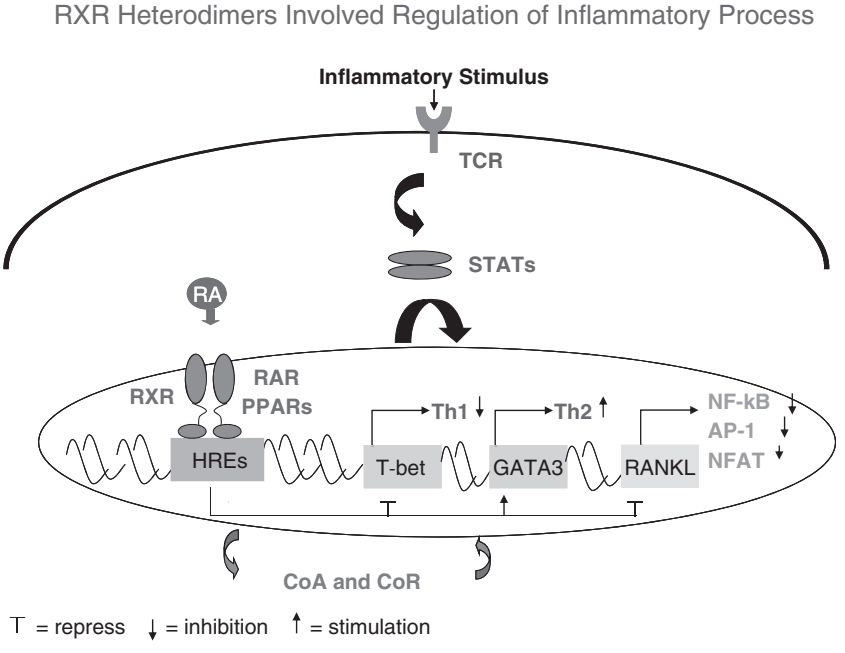


Figure 8.3 Mechanisms of the nuclear receptors regulating the transcription of the inflammatory process. Once the nuclear receptors (such as RXR/RAR and RXR/PPARs) bind with DNA sequences, they start to regulate transcription via the hormone response elements (HREs) which are located in regulatory regions of target genes. The effects of nuclear receptors on transcription are mediated by recruitment of co-regulators including co-activators (CoA) and co-repressors (CoR). Accordingly, the inflammatory process which includes expression of transcription factors and activity of signaling pathways can be modulated by activation of nuclear receptors. RAR and RXR regulate gene transcription after being stimulated by atRA and 9cRA, respectively. Both atRA and 9cRA are derivatives of vitamin A. Thus, administration of vitamin A or its precursor can contribute to the regulation of inflammatory signal transductions.

Accordingly, the anti-inflammatory properties of PPARs is elicited through interfering with various stress signaling pathways and repressing expression of transcription factors such as NF-κB, AP-1, NFAT, and STAT-1 (Cabrero et al., 2002; Genolet et al., 2004). Futhermore, PPARs were shown to reduce the expression of the transcription factor of T cells (T-bet) and activity of the mitogen-activated protein kinase (MAPK) cascade (Jones et al., 2003). Thus, PPARs can contribute to modulating inflammatory responses and reducing the development of autoimmune diseases by several mechanisms.

The activity of PPARs depends on their heterodimer formation with RXR. After activation of the PPAR/RXR heterodimer via their ligands, the transcriptional regulation of PPARs can be initiated through binding to their peroxisome proliferator response elements (PPREs) in the promoter region of target genes. The PPARγ/RXR heterodimer is widely distributed in the mammalian intestinal tract and other organs. The activation of both nuclear receptors PPARγ and RXR may inhibit the transcription of inflammatory cytokines and reduce chronic inflammation. Present studies suggest that retinoid-induced activation of RXR may synergistically stimulate PPAR responses and enhance PPAR transcriptional regulation of inflammatory mechanisms (Kamenov et al., 2004; Ahmed et al., 2007). Desreumaux et al. (2001) demonstrated that activation of the PPARγ/RXR heterodimer

through their ligands such as free fatty acids or 9-cis RA protected against trinitrobenzene sulfonic acid (TNBS)-induced intestinal inflammation and prevented the development of colitis in mice. They also demonstrated that RXR agonists were equally effective as PPARγ agonists in mitigating intestinal inflammation. RXR agonists are primarily derived from the retinoid and carotenoid dietary supplementation. Accordingly, retinoids and carotenoids contribute to the activation of RXR ligand. Therefore, the carotenoid- and retinoid-based nutritional intervention may have the synergistic anti-inflammatory effects on inhibiting the stress signaling pathways in chronic intestinal inflammation.

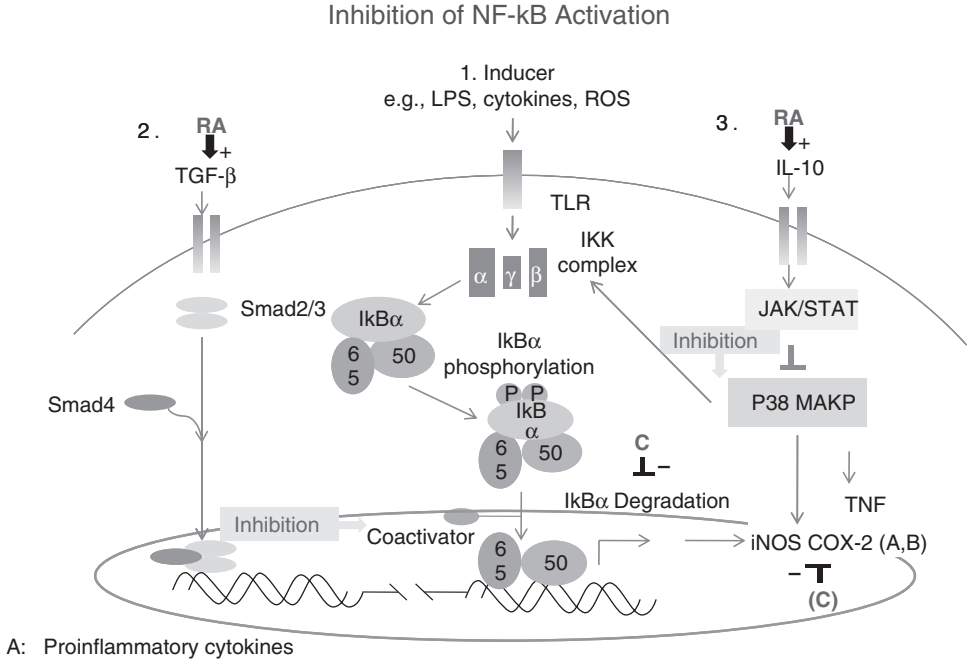
The nuclear receptor superfamily, including steroid, T3, retinoid, and orphan nuclear receptors is a group of ligand-dependent transcriptional regulatory proteins which have functional effects on the development of the immune system. Current evidences indicate that the nuclear receptor family plays a key role in the suppression of proinflammatory process (Desreumaux et al., 2001; Winoto and Littman, 2002; Kamenov et al., 2004). The orphan nuclear receptors such as Nur77 (nerve growth factor IB or NR4A1, nuclear receptor subfamily 4 group A member 1) have been implicated in T-cell apoptosis and tumor cell proliferation (Kang et al., 2000). Nur77 can interfere with Bcl-2 which is known to protect activated T cells from apoptosis. However, Nur77 translocation and function are tightly controlled by RAR and RXR (Winoto and Littman, 2002). Kang et al. (2000) demonstrated that transcriptional activity of Nur77 was significantly inhibited by co-transfection of RAR or RXR. This is also the possible mechanism by which RA can inhibit T-lymphocyte-mediated apoptosis.

RAR, RXR, and PPAR are becoming potential therapeutic targets used to treat T-cell-mediated autoimmune diseases as well as cancers. For instance, the RXR agonist-induced synergistic activation of PPARγ has been demonstrated to reduce the colon inflammation (Desreumaux et al., 2001). Thus, co-administration of different nuclear hormone receptor agonists may be a potential therapeutic strategy to mitigate T-cell-mediated autoimmune disease such as IBD. The retinoids and carotenoids include several potential agonists that can activate retinoid receptors through specific binding. The administration of retinoids and carotenoids may synergistically regulate the gene expression to reduce proinflammatory events of autoimmune diseases. However, the biological role of retinoid receptors in the maintenance of mucosal immune homeostasis is not fully understood, even though the anti-inflammatory effects of RXR have been identified in colon inflammation. The health benefits of carotenoids and retinoids still need to be addressed, which can lead to the development of new approaches for prevention of inflammatory immune disorders.

8.6 EFFECTS OF RETINOIDS AND CAROTENOIDS ON THE OXIDATIVE STRESS SIGNALING PATHWAY

The ROS generated during the phase of inflammatory response may act as signaling molecules leading to the modulation of multiple gene expression which is related to phagocytosis, immune cell proliferation, and apoptosis. The extracellular ROS also can induce a change in the phosphorylation status of transcription factors which result in the activation of particular signaling cascades. Constant activation of these signaling cascades leads to the disturbance of cellular redox balance as well as dysregulation of inflammation responses. In pathological conditions of IBD, the inflammatory responses are mediated by a number of stress-associated kinase pathways including JNK/p38 MAPK and redox-sensitive tran-

scription factors (NF-κB). The dysregulated activation of NF-κB via TLRs may be a result of NOD2 gene mutation which is highly correlated with pathogenesis of IBD. NF-κB is able to stimulate the production of proinflammatory molecules such as NO and prostaglandins synthesized by iNOS and COX pathways, thereby enhancing the severity of inflammation (Baniyash, 2006; Werner and Haller, 2007). Generally, NF-κB plays a crucial role in regulating immune responses to infection. NF-κB enhances the expression of genes related with proinflammatory cytokines, enzymes, and adhesion molecules, as well as production of ROS in chronic inflammatory disease such as IBD (Schottelius and Baldwin, 1999). The proinflammatory cytokines such as TNF-α and oxidative stress generated during the inflammation phase can in turn promote the activation of the NF-κB. The innate mechanism of NF-κB activation and the production of proinflammatory factors in IBD are summarized in Figure 8.4.



A: Proinflammatory cytokines

B: Cellular adhesion molecules

C: Beta-carotene

RA: Retinoic acid

Figure 8.4 Schematic diagram explaining the mechanisms involved in the activation of NF-κB, as well as regulating the effects of beta-carotene and retinoic acid on NF-κB activation. (1) The pathway explains the activation process of IkB/NF-κB system. P65 is also a protein also termed RelA, a subunit of transcription factor NF-κB. Beta-carotene can inhibit IkBα degradation and production of iNOS, COX-2, and proinflammatory cytokines. (2) The pathway demonstrates the Smad signal transduction cascade activated by TGF-β, which suppresses the NF-κB-mediated proinflammatory gene expression. RA can enhance the expression of TGF-β. (3) The pathway demonstrates IL-10-activated signal transduction cascade which may suppress the proinflammatory signal cascade associated with NF-κB through inhibiting the p38 MAPK activation. The IL-10 may inhibit p38 MAPK pathway by activating the JAK/STAT pathway (adapted and redrawn from Kontoyiannis et al., 2001).

The activity of NF-κB involved in chronic inflammation of IBD has been analyzed by several studies. Schreiber et al. (1998) identified the increased level of NF-κB and p65, which is a subunit of the NF-κB complexes, in the *lamina propria* (LP) biopsy specimen from CD patients. LP is a vital part of the GALT, which contains large members of T cells. Rogler et al. (1998) determined that the activation of NF-κB was significantly increased in the inflamed mucosa. In a recent study, the enhanced NF-κB expression was found in inflamed mucosal biopsies of children suffering from CD (Negroni et al., 2006). Therefore, the suppression and regulation of NF-κB activation are promising approaches to modulate the IBD progression.

Since the transcription factor NF-κB is very sensitive to oxidative stress (OS), ROS generated under the OS conditions might play a key role in modulating the dysregulation of immune responses in IBD in addition to causing cellular damage. The chemopreventive activities of the carotenoids can quench ROS generated during the inflammation phase and potentially modulate the perpetuating stimulation of NF-κB pathway in IBD. Pavlick et al. (2002) concluded that the overproduction of NO from upregulated iNOS induced chronic colitis in IBD. Mannick et al. (1996) determined that beta-carotene supplementation resulted in a significant reduction of iNOS in patients with *Helicobacter pylori* infection. Bai et al. (2005) comprehensively analyzed the effects of beta-carotene on the redox-based NF-κB activation in the LPS-stimulated macrophages. The results from their study showed that beta-carotene inhibited iNOS and COX-2 expression, reduced the production of pro-inflammatory cytokines TNFγ and IL-1β, and suppressed NF-κB activation by inhibiting degradation of IkB-α. Palozza et al. (2003) established that beta-carotene played a vital role in the redox regulation of NF-κB activation, which is involved in the balance between promoting proliferation and inhibiting apoptosis of cells. Thus, the carotenoid intervention can potentially regulate the redox status of NF-κB activation in the IBD progression (Fig. 8.4). In addition, since the retinoids may interact with anti-inflammatory transcription pathways to reduce inflammatory gene expressions, RA and dietary vitamin A may contribute to an increase in the levels of TGF-β and IL-10 which can lead to the suppression of persistent immune responses in IBD. The current studies suggested that TGF-β and IL-10 influenced NF-κB signaling cascade and suppressed proinflammatory gene induction thereby regulating intestinal epithelial cell homeostasis as shown in Figure 8.4 (Schottelius and Baldwin, 1999; Kontoyiannis et al., 2001; Werner and Haller, 2007). However, the regulating effects of carotenoids and retinoids on molecular mechanisms of NF-κB activation in IBD are not well defined. Further studies are required to analyze the influence of carotenoids and retinoids on inflammatory gene expression during the IBD progression. This will be beneficial for a comprehensive understanding of the potential biological role of dietary carotenoids in IBD, which are primary precursors of retinoids for humans and animals.

As novel intervention strategies, carotenoids and retinoids contribute to reducing the production of proinflammatory cytokines and suppressing the activity of proinflammatory signaling cascades. Administration of carotenoids may also contribute to inhibiting the activation of the oxidative stress-associated signal transduction. In addition, a long-term supplementation with vegetable juice, which contains an abundance of carotenoids, may be beneficial for preventing intestinal chronic inflammation and the development of IBD as well as colon cancer. In addition to carotenoids, polyphenols show modulatory effects on inflammatory responses (Gonzalez-Gallego et al., 2010) and thus, consumption of a variety of foods enriched in carotenoids and polyphenols will be a strategy to prevent or reduce inflammatory diseases.

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9 Ruminant *Trans* Fat as Potential Nutraceutical Components to Prevent Cancer and Cardiovascular Disease

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9.1 INTRODUCTION

The implication of *trans* fatty acids (TFA) has received increasing attention over the last few years due to potential public health concerns. Specifically, the scientific literature has begun to differentiate between *trans* fat produced by ruminants (ruminant trans fatty acid [rTFA]) by "a natural biohydrogenation reaction," and those produced as a by-product of industrial processing (industrial *trans* fatty acid [iTFA]). Collectively, there is now substantial evidence to support the notion that rTFA (found in dairy and beef produce) and iTFA (synthetically produced oils) have very disparate implications for health and putative bioactivty. In this review, we consider the most recent findings from human, animal, and cell culture studies in order to discuss the health implications of rTFA in the development of different types of cancer and cardiovascular diseases (CVDs), as well as evaluate the potentials of developing rTFA as novel components of functional food.

TFAs refer to a class of fatty acids that contain one or more double bonds in the *trans* configuration. It is interesting that most of the TFA do not have a "natural" origin, but have been introduced into commercial solid edible fats generated by the partial hydrogenation of vegetable oils (a method developed for the demand of longer shelf life of foods and to replace animal fats, e.g., lard and tallow). Epidemiological data from retrospective case-control, prospective cohort, and nested case-control studies strongly support positive associations between TFA intake and coronary heart disease (CHD) (Willett et al., 1993; Oomen et al., 2001). In particular, the increased CHD risk has been associated with the intake of both total TFA and foods known to contain major sources of TFA such as margarine, cakes, and cookies (Oomen et al., 2001). Further evaluation of CHD risk indices has also revealed that TFA intake from partially hydrogenated vegetable oil (PHVO) was positively associated with higher blood concentrations of inflammatory markers (e.g., C-reactive protein) and impaired endothelial function; reduced insulin sensitivity/secretion; impaired fetal development (anthropometric measures); and infant essential fatty acid status (Innis, 2006; Mozaffarian et al., 2009). When PHVO was replaced with other fat such as vegetable oils,

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there was an estimated 50% reduction in overall CHD risks (Mozaffarian and Clarke, 2009). In 2002, the panel on Macronutrients of the U.S. National Academies of Science, Institute of Medicine, recommended that *trans* fat consumption be as low as possible in a nutritionally adequate diet. Subsequently, in 2003, the World Health Organization recommended that *trans* fat intake be limited to less than 1% of overall energy consumption. As a result, recent consensuses showed a global decline of total TFA consumption from an average of 10 g/day worldwide a decade ago, to 3–4 g/day in North America, 2–4 g/day in northern European countries such as Denmark and the Netherlands, 1–3 g/day in Mediterranean countries, and less than 1 g/day in East Asian countries during the past few years (Hulshof et al., 1999; Craig-Schmidt, 2006).

Since then, complexities of TFA intake have arisen, particularly due to increasing recognition that TFA also occur naturally in various ruminant-based foods such as dairy, beef, and lamb. The two predominant "natural" isomers in ruminant-derived foods include *c*9,*t*11-conjugated linoleic acid (CLA) and *trans*-11 vaccenic acid (VA) (Palmquist et al., 2005). Both CLA and VA fatty acids are produced during the biohydrogenation of C_{18}-polyunsaturated fatty acid (PUFA), primarily linoleic acid and α-linolenic acid, with the presence of rumen bacteria (Lock et al., 2004). Indeed, VA is the major biohydrogenation intermediate that accumulates in the rumen and later in the tissue, while *c*9,*t*11-CLA is present mainly as a transitory product. It is estimated that over 60% of *c*9,*t*11-CLA in ruminant fat is endogenously synthesized by Δ9-desaturase using VA as the substrate (Griinari et al., 2000). Traditionally, rTFA has been considered to constitute a rather small part of the fat in dairy products (2–5% of total fatty acids) and beef and lamb (3–9%) (Precht and Molkentin, 1996). However, we now appreciate that the fatty acid composition is largely dependent on the bovine feeding practice and can vary substantially due to geographical and/or seasonal change. For example, alpine pastures in Northern European countries yield greater concentration of rTFA than lowland pastures (Precht, 1995). Milk produced during the long daylight of summer often contains more total rTFA than milk produced during the winter months (Precht, 1995). Moreover, modification to agricultural practices have been developed to enhance the content of rTFA, such as supplementing feed with oil seeds (Rego et al., 2009), changing the microflora of the rumen (Boeckaert et al., 2008), and preselecting cows that are genetically high "rTFA producers" (Taniguchi et al., 2004). As a consequence, TFA content as high as 12% of total fat is now a regular occurrence in dairy products (with additional reductions in saturated fat) (Palmquist et al., 2005; Perfield et al., 2007; Mendis et al., 2008). The increase in the proportion of rTFA in dairy-derived products has confounded the premise for minimizing total dietary TFA and sparked a critical need to better understanding the bioactivity of specific rTFA isomers (e.g., VA).

While there seems to be considerable evidence supporting a positive association between TFA intake and CHD risk, very few studies have attempted to differentiate between iTFA and rTFA. There is, however, a limited number of epidemiological studies that imply a positive association with CHD risk only exists between TFA isomers generated by industrial means (e.g., C18:1 and C18:2) and not those rTFA isomers formed through biohydrogenating reactions such as *trans*-C16:1 (Mozaffarian et al., 2004, 2005; Lopez-Garcia et al., 2005). Interestingly, there has been some acknowledgment that rTFA isomers (e.g., CLA) have different health effects than most of the PHVO-derived iTFA. Recently, the definition of TFA in the Codex Alimentarius standard and official dietary recommendations of countries such as the USA, Canada, and Denmark have been amended to exclude TFA isomers with conjugated *trans* double bonds. Despite the recognition that some conjugated

TFA may have differential biological effects for disease risk, this is not inclusive of all rTFA isomers, nor is it inclusive of the most predominant rTFA, *trans*-11 VA.

We hereby review well-controlled intervention studies in human population and experimental models, with a specific focus on purified synthetic rTFA isomers (i.e., *c*9,*t*11-CLA and VA) as well as dairy products enriched with VA and CLA, in order to generate a clear understanding of the current progress on rTFA research and the feasibility of related nutraceutical application.

9.2 *C9,T11*-CLA ISOMER AND HEALTH IMPLICATIONS

CLA refers to a group of geometrical and positional isomers of linoleic acid [*c*9*c*12-18:2(n-6)], of which, about 80% is the *c*9,*t*11 isomer. When CLA is commercially made from vegetable oils, such as those found in retail supplements, it is usually composed of a mixture of isomers, predominantly *c*9,*t*11- and *t*10,*c*12-CLA. In the absence of supplementation, one's intake of CLA is dependent almost exclusively on the amount of ruminant fat consumed. The average intake of CLA varies considerably, ranging from as low as 0.1 g/day to as high as 1.5 g/day (15-fold difference) due to distinctive dietary patterns and variations in fatty acid composition of dairy products. For instance, the intake of the *c*9, *t*11 isomer by a group of healthy males in Western Canada was reported to be at the lower end of published reports (95 mg/day) (Ens et al., 2001), and similar to the estimated intake of Americans (Ritzenthaler et al., 2001), whereas the average daily intake of *c*9,*t*11 isomer in Australians has been reported to be as high as 1500 mg (Parodi, 2003).

More recently, it has been recognized that the endogenous *in vivo* synthesis of *c*9,*t*11-CLA may be the greater contributor to whole body concentration of this isomer compared to luminal synthesis in ruminant animals (Palmquist et al., 2005). In humans, the conversion of VA to *c*9,*t*11-CLA has been estimated to range from 11% to 30% (Turpeinen et al., 2002). Due to the complex origins of this isomer, it is possible that the existing estimation of dietary intake of *c*9,*t*11-CLA alone, regardless of its accuracy, may not adequately represent the total pool *in vivo*, as an increased dietary intake of VA would also add cumulatively to the putative abundance of *c*9,*t*11-CLA in the body.

9.2.1 CLA modulates carcinogenesis

CLA has been reported to affect initiation, promotion, and metastasis of mammary/breast, prostate, and gastrointestinal cancers in experimental animal models (either carcinogen induced or genetically modified). Although several isomers of CLA have been shown to have antitumorogenic properties which has been discussed elsewhere (Kelley et al., 2007), we have selectively focused our discussion on the natural *c*9,*t*11 isomer so as to evaluate its nutraceutical value (Table 9.1).

9.2.1.1 CLA and breast cancer

Young female Sprague Dawley rats injected with carcinogens such as methylnitrosourea (MNU), dimethylbenz[a]anthracene (DMBA), and benzo[a]pyrene (BP) are a class of well-developed carcinogenesis models with detectable premalignant lesion in mammary gland within a few weeks after carcinogen administration (Mehta, 2000). Dietary *c*9,*t*11-CLA (0.5–1%) treatment has been shown by different research groups to effectively reduce the

Table 9.1 Summary of studies examining the effect of rTFA on several types of cancer

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Mammary cancer				
Banni et al. (2001)	Conversion of VA to CLA Anticancer effect of VA compared to CLA	*Study 1*: Healthy rats were fed diets containing 0, 1, 2, 3% w/w VA (purified) for 3 weeks. *Study 2*: Rats with MNU-induced mammary tumor were fed for 6 weeks. 1. 2% w/w VA 2. 1% c9,*t*11-CLA.	*Study 1*: VA ↑ CLA (c9,*t*11) levels in mammary gland and liver. Maximum CLA production at 2% w/w. *Study 2*: VA (2% w/w) ↓ mammary tumors similar to c9,*t*11-CLA (1% w/w).	Anticancer response to VA is mediated by its conversion to CLA.
Corl et al. (2003)	Conversion of VA to CLA and the ability of VA to inhibit the development of mammary carcinomas.	Rats with MNU-induced mammary tumor were fed for 24 weeks. 0.13–1.6% w/w VA, 0.05–0.37% w/w c9,*t*11-CLA (from butter).	↑ tissue CLA content with ↑ dietary VA. ↓ tumor formation in mammary gland	Dose-dependent effect of VA + CLA on carcinogenesis.
Ip et al. 1999	Compare the bioactivity of c9,*t*11-CLA in dairy food or as free fatty acids and the bioactivity of different CLA isomers	Rats with MNU-induced mammary tumor were fed for 4 weeks. 1. Control butter (0.1% CLA) 2. High CLA butter (0.8% CLA) 3. c9,*t*11-CLA (0.8% CLA) 4. CLA mixture (0.8% CLA)	All CLA diets↓ tumor cell number and proliferation. CLA mixture and CLA butter diet showed similar efficacy. Selective uptake of c9,*t*11 over *t*10,c12 isomer.	Milk fat CLA reduce mammary cancer risk. C9t11 isomer is as potent as mixture. High bioactivity in CLA butter is due to additional endogenous CLA produced from VA
Lock et al. (2004)	Determine reduced carcinogenesis with VA feeding is directly mediated via conversion to c9,*t*11-CLA.	Rats with MNU-induced mammary tumor were fed for 6 weeks. 1. 0.13% VA 2. 0.4% VA 3. 1.6% VA 4. 1.6% VA + Δ^9 desaturase inhibitor (sterculic oil)	1.6% VA ↑ CLA content in tissues and ↓ tumor growth. 1.6% VA + Δ^9 desaturase inhibitor had ↑ CLA liver and plasma concentrations than low VA groups. Inhibitor reversed effects of VA.	The anticarcinogenic effect of VA is predominantly, perhaps exclusively, mediated through its conversion to c9,*t*11-CLA via Δ9-desaturase.
Ip et al. (2002)	Assess differences in anticancer activity of c9,t11- and t10,c12-CLA isomers	Rats with MNU-induced mammary tumor were fed for 6 weeks. 0.5% w/w of either purified CLA isomers	Lower tissue *t*10,c12-CLA than c9,*t*11-CLA. ↓premalignant lesions by both isomers but no difference between groups	Anticancer efficacies of the two isomers were similar.

Lavillonnière et al. (2003)	Determine bioactivity of *c*9,*t*11-CLA on mammary tumor growth compared to CLA mixture	Rats with MNU-induced mammary tumor were fed for 20 weeks. 1. 1.0% w/w sunflower oil 2. 1.0% w/w *c*9,*t*11-CLA, purified 3. 1.0% CLA mixture	↓ tumor mass per rat in both CLA groups Incidence and latency unaffected by diet	*C*9,*t*11-CLA has anticarcinogenic properties in experimental mammary carcinogenesis.
Ip et al. (2001)	Effect of CLA mixture and *c*9,*t*11-CLA from butterfat on cell cycle regulatory protein and proliferative activity of developing mammary epithelium in rats.	*Study 1*: Healthy rats were fed basal diet or 1% CLA mixture for 4 weeks. *Study 2*: Healthy rats were fed control butter (0.1% w/w) or CLA-butter (0.8% w/w) for 4 weeks. *Study 3*: Healthy rats were fed basal diet or 1% purified *c*9,*t*11-CLA for 2, 4, 6, 8 weeks.	↓ proliferative activity, cyclin D1 and cyclin A in 3 CLA groups. Highly proliferative cells are more responsive to CLA intervention.	CLA mixture and *c*9,*t*11-CLA from butter are equally effective. Responsiveness to CLA is dependent on proliferative status.
Hubbard et al. (2007)	Effect of CLA mixture on matrix-modifying proteins within metastatic mammary tumor cells	Mice transplanted with metastatic tumor cells in mammary fat pad after 3 weeks were fed 0, 0.1, 0.5, 1.0% w/w CLA mixture.	↑ expression of TIMP-1&2 [matrix metalloproteinase (MMP) inhibitor]	Suppression of MMP activity may be a pathway through which CLA reduces tumor invasion and spread.
Ip et al. (2007)	Effect of two major CLA isomers in a clinically relevant breast cancer model	Transgenic mice overexpressing erbB2 in mammary epithelium were fed with 0.5% w/w purified *c*9,*t*11-CLA or *t*10,*c*12-CLA from days 68–72 till tumor reached a size of 18–20 mm in the larger diameter.	*T*10,*c*12-CLA ↑ mammary tumor development and metastasis while *c*9,*t*11-CLA did not.	*T*10,*c*12-CLA stimulates mammary tumorigenesis in this model, but might prevent cancer in rat models.
Russell et al. (2007)	The anticancer effect of CLA is related to mass cell-derived cytokines in mammary gland stromal remodeling.	Healthy mice *Exp 1*: 0.1%, 2% CLA mixture for 1, 3, 5, or 7 weeks *Exp 2*: 0, 0.5%, 1% purified *c*9,*t*11- or *t*10,*c*12-CLA isomer for 3 days, 7 days, or 7 weeks *Exp 3*: 0 or 0.5% *t*10,*c*12-CLA for 7 days in mice with or without functional mass cells	CLA mixture or *t*10,*c*12 isomer showed effect on stromal remodeling while *c*9,*t*11-CLA did not. TNF-α or mass cells are not required for the remodeling.	*T*10,*c*12-CLA induces inflammatory and fibrotic phenotype in the mammary gland independent of TNF-α or mass cells.

Continued

Table 9.1 *Continued*

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Hubbard et al. (2003)	Effect of *c*9,*t*11-and t10c12-CLA on mouse mammary tumor metastasis	Mice were transplanted with metastatic tumor cells in mammary fat pad after 3 weeks on diet, and then were fed for additional 4 weeks. 1. no CLA 2. low c9,*t*11-CLA (0.1%) 3. high *c*9,*t*11-CLA (0.25%) 4. low *t*10,*c*12-CLA (0.1%) 5. high *t*10,*c*12-CLA (0.25%) 6. CLA mixture (0.125% each)	No effect on latency or growth of primary tumor line by separate isomers or mixture. ↓ tumor burden and metastasis by separate isomers and mixture in a dose-dependent manner.	Two CLA isomers have similar antitumor efficacy and might share similar mechanisms for decreasing tumor burden in this model. No additive effects for two isomers.
Chujo et al. (2003)	Effect of CLA isomers on growth factor (GF)-induced breast cancer cell proliferation	MCF-7 cells induced with growth factor were treated with 1. control 2. *c*9,*t*11-CLA (10 μM) 3. *t*10,*c*12-CLA (10 μM) 4. CLA mixture (10 μM)	Both isomers and mixture ↓ cell proliferation, with *c*9,*t*11-CLA the most potent. Only *t*10,*c*12–CAL inhibited GF-induced proliferation.	Both CLA isomers can inhibit MCF-7 cell proliferation, but have separate mechanisms and different targets of actions.
Tanmahasamut et al. (2004)	Whether CLA has direct antiestrogenic activity or interfere with estrogen signaling in ER+ breast cancer cells	MCF-7 were cells treated with five individual CLA isomers and CLA mixture at 25–200 μM for 2 days.	CLA treatment ↓ ERα expression and estrogen response element activity. C9,*t*11-CLA is the least potent.	CLA compounds possess potent antiestrogenic properties on breast cancer cells.
Miller et al. (2001)	Effect of CLA on growth and arachidonic acid (AA) metabolism in human breast cancer cells	MCF-7 cells were treated at 17.8 μM or 57 μM for 24 hours or 4 days. 1. CLA mixture; 2. *c*9,*t*11-CLA; 3. *t*10,*c*12-CLA; 4. linoleic acid	C9,*t*11-CLA ↑uptake of AA into MAG and AA conversion to PGF_2PE ↓uptake into PC and AA conversion to PGE_2	CLA isomers suppress growth by affecting AA distribution among cellular lipids and altering prostaglandin profile.
Kim et al. (2005)	Effect of CLA on mouse mammary tumor cell growth and lipoxygenase pathway	Mouse mammary tumor cell line 4526 were treated for 24, 48, and 96 hours at 10–100 μM 1. *c*9,*t*11-CLA; 2. *t*10,*c*12-CLA 3. LA	Both *t*10,*c*12-CLA and a lipoxygenase inhibitor, but not *c*9,*t*11-CLA or LA, ↓ tumor cell proliferation and induced apoptosis.	*T*10,*c*12-CLA, not *c*9,*t*11-CLA, reduces tumor cell growth by suppressing lipoxygenase metabolite production.

Degner et al. (2006)	Effect of CLA on COX-2 transcription in breast cancer cells	MCF-7 cells were treated with *c*9,*t*11-, *t*10,*c*12- CLA or CLA mixture at 20, 40, or 80 μM for 24 hours	CLA purified isomers and mixture ↓ nuclear regulatory proteins involved in AP-1 binding and COX-2transcription. *t*10,*c*12-CLA is more effective than *c*9,*t*11-CLA.	CLA attenuate COX-2 transcriptional activity via reducing activator protein-1 binding.
Kemp et al. (2003)	Effect of CLA on cell cycle control in breast cancer cells	MCF-7 cells were treated with *c*9,*t*11-, *t*10,*c*12- CLA or CLA mixture at 10–160 μM for 24, 48, or 72 hours	↓ cyclins D1 and E required for G1 to S-phase transition ↑ tumor suppressor p53, p27& p21	Antiproliferative effect of CLA is mediated via cell cycle control. *T*10,*c*12-CLA is more effective than *c*9,*t*11-CLA.
Miller et al. (2003)	Conversion of VA to *c*9,*t*11-CLA in breast cancer cells	MCF-7 cells were treated with 5–20 μg/mL *c*9,*t*11-CLA for 4 days	VA increased *c*9,*t*11-CLA in a dose-dependent manner. VA↓cell growth and ↑ DNA fragmentation (at 20 μg/mL only)	VA suppresses mammary cancer cell growth likely by conversion to *c*9,*t*11-CLA.
Amaru and Field (2009)	Mechanism of CLA's effect on breast cancer cells	MCF-7 cells were treated with *c*9,*t*11-CLA, *t*10,*c*12-CLA, linoleic acid, or oleic acid at 128 or 256 μM for 24 or 48 hours	Both isomers ↓ metabolic activity of IGF-1 stimulated cells, IGF-1 receptor, and insulin receptor substrate-1, but *t*10,*c*12-CLA is more potent.	*T*10,*c*12-CLA rather than *c*9,*t*11-CLA inhibits breast cancer cell growth via interfering with IGF-1 and related signaling pathways.
O'Shea et al. (2000)	Effect of milk fat enriched with CLA on growth and antioxidant enzyme defense system in human breast cancer cells	MCF-7 cells were treated with milk fat yielding CLA at 16.9–22.6 ppm for 8 days.	↓ cell growth, cell viability ↑ peroxidation (superoxide dismutase, catalase, and glutathione peroxidase activities)	Milk fat TG-bound CLA, primarily *c*9,*t*11-CLA, was toxic in this breast cancer cell line.
Gastrointestinal cancer				
Mandir and Goodlad (2008)	Effect of CLA isomers on polyp development in intestinal cancer	*Apc*$^{min/+}$mice were fed for 6 weeks with 1. control diet 2. 1% *c*9,*t*11-CLA 3. 1% *t*10,*c*12-CLA 4. 0.5% *c*9,*t*11-CLA + 0.5% *t*10,*c*12-CLA	↓ polyp number ↓ colonic weight and crypt fission length only by *c*9,*t*11-CLA ↑polyp diameter and intestine weight by *t*10,*c*12-CLA	*C*9,*t*11-CLA is more protective than other isomers.

Continued

Table 9.1 *Continued*

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Soel et al. (2007)	Effect of CLA isomers on the metastasis of colon cancer cells *in vitro* and *in vivo*	BALB/c mice injected with tumor cells were fed no CLA or 0.1% *c*9,*t*11-or *t*10,*c*12-CLA for 4 weeks. SW480 colon cancer cells treated with no CLA or *c*9,*t*11-or *t*10,*c*12-CLA at 1,2 or 4 μM for 24 hours	Both CLA isomers reduced pulmonary nodule number *in vivo*. Only *c*9,*t*11-CLA inhibited cell migration and MMP-9 activity *in vitro*.	*C*9,*t*11-CLA is more effective in inhibiting colon cancer metastasis. *T*10,*c*12-CLA might act via other mechanisms.
Rajakangas et al. (2003)	Effect of CLA isomers on intestinal carcinogenesis	Min mice were fed control diet or 1% purified *c*9,*t*11- or *t*10,*c*12-CLA for 8 weeks.	No effect on adenoma number, but *t*10,*c*12-CLA ↑adenoma size in distal intestine, lipid peroxidation, and cyclin D1 expression, ↓ nuclear NF-κB	*T*10,*c*12-CLA can act as a cancer promoter in colon carcinogenesis whereas *c*9,*t*11-CLA showed no adverse effect.
Beppu et al. (2006)	Compare the effect of four CLA isomers on the growth of human colon cancer cell lines.	Caco-2, HT-29, and DLD-1 cells were treated with 200 μM of *c*9,*c*11-, *c*9,*t*11-, *t*9,*t*11- or *t*10,*c*12-CLA for 24, 48, or 72 hours	All isomers reduced cell viability and DNA fragmentation in a time-dependent manner, with *c*9,*t*11-CLA being less effective than others.	*C*9,*t*11-CLA has weak anticarcinogenic effect in human colon cancer cells.
Palombo et al. (2002)	Compare antiproliferative effect of CLA isomers and commercial preparations in human colorectal cells.	HT-29 and MIP-101 cells were treated with CLA mixtures (178, 100, and 36 μM) or purified *c*9,*t*11, *t*10,*c*12, or *c*9,*c*11 isomers (100 and 50 μM) until the cells in the control wells reached 60–70% confluence.	Dose-dependent reduction in cell proliferation by CLA mixtures or individual isomers in both cell lines	*C*9,*t*11-CLA has modest antiproliferative properties in colon cancer cells.
Cho et al. (2006)	Effect of CLA isomers on cell cycle and cell cycle regulatory proteins in colon cancer cells	HT-29 cells were treated with purified *c*9,*t*11 or *t*10,*c*12 CLA at 0, 1, 2, or 4 μM	Only *t*10,*c*12-CLA induced G1 arrest by modulating p21, retinoblastoma protein phosphorylation pathways	*c*9,*t*11-CLA has no effect on the cell cycle in this colon cancer cell line.
Lampen et al. (2005)	Cellular and molecular effect of *c*9,*t*11-CLA/VA on colon cancer cells	HT-29 and Caco-2 cells were treated with various concentrations of *c*9,*t*11-CLA and VA (5–200 μM).	↓proliferation via inhibiting c-myc, cyclin D1 and c-jun, and increasing PPAR-δ expression by *c*9,*t*11-CLA in both lines ↓ PPAR-δ expression by VA	*c*9,*t*11-CLA has antiproliferative effect due to the downregulation of APC-β catenin pathway and PPAR-δ signaling.

Liu et al. (2002)	Effect of *c*9,*t*11-CLA on apoptosis of gastric cancer cells	SGC-7901 cells were treated with *c*9,*t*11-CLA at 25–200μM for 24 and 48 hours.	↓ cell growth, apoptotic rate, and proliferative proteins bcl-2 and ki67 in a dose- and time-dependent manner	C9,*t*11-CLA inhibits gastric cancer cell growth and proliferation via blocking cell cycle pathways.
Awad et al. (1995)	Effect of major C18:1 fatty acid on growth and FA composition in human colon cancer cells	HT-29 cells were treated with 18:0, t9-18:1, c9-18:1, t11-18:1, c11-18:1 at 30μM for 9 days.	c11-18:1 and t11-18:1 inhibited cell growth and increased inositol phosphate production.	VA inhibits colon cancer cell growth, possibly via affecting phosphoinositide cycle and membrane lipids.
Miller et al. (2003)	Conversion of VA to *c*9,*t*11-CLA in colon cancer cells	SW-480 cells were treated with 5–20μg/mL VA for 4 days	VA increased c9,*t*11-CLA in a dose-dependent manner. VA↓cell growth, cytosolic GSH, and ↑ DNA fragmentation (at 20μg/mL only).	VA suppresses colon cancer cell growth and induces apoptosis likely by conversion to *c*9,*t*11-CLA.
Reynolds et al. (2008)	Effect of VA and *c*9,*t*11-CLA on human colon cancer cells	Caco-2 cells were treated with VA and *c*9,*t*11-CLA at 50μM for 7 days.	Altered fatty acid profile and stearoyl-CoA desaturase activity by both VA and CLA. CLA but not VA ↓ mRNA levels of TNF-α and IL-6.	VA and *c*9,*t*11-CLA induces SCD desaturation indices and fatty acid profile change, but only CLA affects inflammatory response
Prostate cancer				
Ochoa et al. (2004)	Antiproliferative effect of CLA isomers and commercial preparations on human prostatic carcinoma cells	PC-3 cells were treated with *c*9,*t*11, *t*10,*c*12 CLA or the mixture at 25–150μM for 24 hours.	↓5-LOX and COX-2 by *c*9,*t*11-CLA ↓cell proliferation by both isomers but *t*10,*c*12-CLA was more effective ↓ bcl-2 by t10c12-CLA	C9,*t*11 isomer affects AA metabolism, *t*10,*c*12 isomer modulates apoptosis and cell cycle.
Palombo et al. (2002)	Compare antiproliferative effect of CLA isomers and commercial preparations in human prostate carcinoma cells	PC-3 cells were treated with CLA mixtures (178, 100, and 36μM) or purified c9,*t*11, *t*10,*c*12, or *c*9,*c*11 isomers (100 and 50μM) until the cells in the control wells reached 60–70% confluence.	Modest antiproliferative effect by both isomers ↑caspase-dependent apoptosis by t10c12-CLA	C9,*t*11-CLA has modest antiproliferative properties in prostate cancer cells in a dose-dependent manner.

number of premalignant lesions, proliferation rate of epithelial cells, as well as apoptosis of preneoplastic lesions in these induced cancer models (Banni et al., 2001; Ip et al., 2002; Cheng et al., 2003; Lavillonnière et al., 2003). The inhibition of proliferation by CLA was normally accompanied by reduced terminal end bud (TEB) (the site of tumor formation in both rats and humans) size and density, probably via modulating cell cycle regulators such as cyclin A and cyclin D1 (Ip et al., 2001). In a transplantable mouse model with mammary tumor with spontaneous lung metastasis, CLA reduced the metastasis rate, total tumor burden, and the survival of metastatic cells in a dose-dependent manner without affecting primary tumor growth (Hubbard et al., 2007). However, several other studies have demonstrated no effect of *c*9,*t*11-CLA especially on latency in mice-bearing mammary tumors (Hubbard et al., 2003; Ip et al., 2007; Russell et al., 2007). Evidence from cell culture studies has also failed to reach consensus regarding the anticancer properties of *c*9,*t*11-CLA. *C*9,*t*11-CLA has shown a strong inhibitory effect on MCF-7 cells, an estrogen receptor (ER) positive breast cancer model (Chujo et al., 2003), but not in ER-negative MDA-MB-231 cells (Tanmahasamut et al., 2004), suggesting that ER may play a vital role in mediating CLA effect of mammary tumor. Miller et al. attributed the growth-suppressing effect of CLA to changes in arachidonic acid distribution among cellular phospholipids and an altered prostaglandin profile (Miller et al., 2001). Tanmahasamut, on the other hand, claimed that the c9t11 isomer was the least potent in inhibiting MCF-7 cell growth among five commercially available CLA isomers (Tanmahasamut et al., 2004). Other researchers have observed similar weak effect of this natural isomer when trying to study the metabolic pathways affected by carcinogenesis (Kemp et al., 2003; Kim et al., 2005; Degner et al., 2006; Amaru et al., 2010).

9.2.1.2 CLA and gastrointestinal cancer

Mandir et al. has used a transgenic model to study gut cancer, the $APC^{MIN/+}$ mouse, and showed that purified *c*9,*t*11-CLA preparations can decrease colonic polyp number without increasing its diameter (Mandir and Goodlad 2008). Similarly, Soel and colleagues have reported that, at low dietary concentrations (0.1% w/w), this natural isomer effectively inhibits metastatic migration of mouse colon cancer cells when injected into BALB/c mice (Soel et al., 2007). However, another group using a similar model found no effect of *c*9,*t*11-CLA on the number of adenomas, nor was there any change in mucosal nuclear factor-kappa B (NF-κB) or cyclin D1 protein mass (Rajakangas et al., 2003). Instead, it was suggested that the synthetic form *t*10,*c*12-CLA showed profound inhibitory impact on tumor progression and regulators of cell cycle. *In vitro* studies using different colon cancer lines (such as HT-29, MIP-101 and Caco-2) tend to advocate *t*10,*c*12-CLA more than *c*9,*t*11-CLA with regard to tumor prevention. Three types of colon cell lines have been compared and suggest a similar weak impact of *c*9,*t*11-CLA on cell proliferation and apoptosis (DNA fragmentation) when compared to other isomers (Beppu et al., 2006). A similar conclusion was made from other studies focusing on individual cell culture models but with different treatment time and dosage (Palombo et al., 2002; Kemp et al., 2003; Cho et al., 2006). Nevertheless, a few studies claim an inverse association between *c*9,*t*11-CLA and tumor progression. In one study, the proliferation and differentiation of both HT-29 and Caco-2 cells were significantly inhibited by *c*9,*t*11-CLA in a dose-dependent manner ranging from 10 μM to 200 μM. Proliferation genes such as c-myc, c-jun, and cyclin D1 were shown to be reduced with treatment; so was peroxisome proliferator-activated receptor (PPAR) δ which modulates tumor development (Lampen et al., 2005). In another study,

apoptosis on gastric adenocarcinoma cell line SGC-7901 was induced by different concentration of *c*9,*t*11-CLA, together with inhibited cell growth and proliferation (Liu et al., 2002). The molecular pathway affected was proposed by the same group to be a direct blockage of cell cycle via bcl-2-associated pathways in mitochondria and fas-associated death domain protein.

9.2.1.3 CLA and prostate cancer

Animal studies that have successfully shown protective effect of CLA against prostate cancer have mainly used a mixture of isomers (*c*9,*t*11- and *t*10,*c*12-CLA) rather than individual preparations. CLA mixture has been reported to decrease PhIP-induced mutagenesis in the prostate of transgenic rats (Yang and Cook, 2003). Earlier studies that used transplanted DU145 cells have shown clear antitumorigenic effects of CLA mixture similar to those observed with breast cancer models when implanted into SCID mice (Cesano et al., 1998). A *in vitro* study using purified *c*9,*t*11 isomer reported moderate antiproliferative effects in PC-3 prostate cancer cell line, similar to that reported for colon cancer lines (Palombo et al., 2002), implying anticancer properties of *c*9,*t*11-CLA either alone or in combination with *t*10,*c*12-CLA.

9.3 MECHANISMS OF CLA ACTION ON CANCER

The anticarcinogenic properties of CLA are substantiated in a variety of animal/cell culture studies. Acknowledging the inconsistent findings from these studies, it appears that the anticarcinogenic effect of CLA may vary across species, with rats being more responsive than mice. The efficacy might also depend on tumor types, stages of tumor development, and/or the dose/duration of treatment. As suggested previously, several potential mechanisms are likely involved including alteration of tumor microenvironment (extracellular matrix), arachidonic acid metabolism (e.g., COX-2, 5-LOX), apoptosis (e.g., bcl-2), cell cycle control (e.g., cyclin A1, cyclin D), cell proliferation (e.g., insulin-like growth factor-1), and nuclear receptors that control these metabolic pathways including PPAR (reviewed in Amaru et al., 2010).

To date, there has not been a clinically controlled feeding trial in cancer patients using purified CLA isomers. One retrospective case-control study suggested an inverse association between serum CLA concentration and breast cancer incidence among postmenopausal women (Aro et al., 2000). On the contrary, in a larger prospective cohort (the TRANSFAIR study), CLA intake showed a weak but positive relation with breast cancer incidence (Voorrips et al., 2002). It is noteworthy that the estimated CLA intake in human population (300 mg/day on average, 0.1% of total energy intake) varies considerably but certainly falls far below the pharmacological doses (1.0% w/w on average, 3–5% of total energy intake) used in either animal or cell culture studies.

9.4 CLA MODULATES CHD RISK FACTORS

CHD has a complex etiology with contribution from both lipid metabolism and inflammatory pathways that cumulatively affect vascular function. Atherosclerosis is considered as a result of hypercholesterolemia, which is defined as elevated concentrations of

circulating low-density lipoprotein (LDL) cholesterol. When oxidized, LDL-cholesterol initiates atheromatous plaque formation through the recruitment of leukocytes (e.g., macrophages) to the intima of the arterial wall. Macrophages subsequently overaccumulate lipids and sterols at the focal site and are eventually transformed into lipid-laden "foam" cells (Ishigaki et al., 2009). Notably, small dense LDL particles tend to be considered more atherogenic due to their higher susceptibility to oxidation, accelerating the accumulation of macrophages (Krauss, 1994; Saha et al., 2009). Plaque formation is also known to be exacerbated by elevated plasma concentration of triglyceride (TG)-rich remnant lipoproteins (including intestinally derived cholesterol-rich chylomicrons) (Proctor et al., 2002). Therefore, circulating cholesterol-rich lipoprotein particles and the rate of cholesterol accumulation at the intima of vessel wall are considered vital indicators of atherosclerosis development.

Feeding CLA, particularly the *c*9,*t*11 isomer, has been reported to improve the blood lipid profile at an average dose of 1.0% w/w (equivalent to 3–5% of energy) in several animal models. In hamsters, plasma TG, total cholesterol, and LDL-cholesterol (especially small dense LDL) concentrations were shown to be lower when fed 0.5–1% *c*9,*t*11-CLA for 12 weeks (Wilson et al., 2006; LeDoux et al., 2007). Data from our own research group has shown similar changes in these parameters in a rat model of the metabolic syndrome that spontaneously develops cardiovascular complications (Proctor et al., 2007; Jacome-Sosa et al., 2009). There have been additional reports that less cholesterol accumulates in the aortic arch and fewer fatty streak lesions develop in CLA-supplemented hamsters (Mitchell et al., 2005; Wilson et al., 2006). Compared to the cholesterol-fed hamster, the apoE-knockout mouse is arguably a better model to study the progression of fatty lesions due to its genetic susceptibility to atherosclerosis (Reddick et al., 1994). When fed 1% *c*9,*t*11-CLA for 12 weeks, the development of atherosclerosis in apo $E^{-/-}$ mice was significantly reduced and associated with reductions in plasma cholesterol and free fatty acids as well as increased (beneficial) apoA-I concentration. There was also significantly less cross-sectional lesion area in the aortic root of treated mice (Arbones-Mainar et al., 2006). In addition to reduced progression, the regression of preestablished atherosclerotic lesions was also reported in the aorta of CLA-fed apo-$E^{-/-}$ mice (Toomey et al., 2003). Similar results have been reported in cholesterol-fed rabbits that had decreased scoring of atherosclerotic lesions in both the thoracic and arch regions of the aorta (Kritchevsky et al., 2004).

A number of double-blind randomized clinical trials have been conducted in overweight but otherwise healthy subjects in an effort to elucidate the effect of *c*9,*t*11-CLA on risk markers of CVD risk (summarized in Table 9.2). One study concluded that *c*9,*t*11-CLA alone had neutral effect on biochemical parameters associated with atherosclerosis and metabolic syndrome, whereas CLA mixture (with various combinations of *c*9,*t*11- and *t*10,*c*12-CLA isomers), tend to be harmful to CVD risk markers. It is worthwhile to note that most of these trials were targeting healthy population with higher CVD risk rather than patients diagnosed with cardiovascular complications per se; therefore, the efficacy of CLA intervention remains largely limited.

9.5 MECHANISMS OF CLA ACTION ON CHD

CLA, more specifically the *c*9,*t*11 isomer, is one of the few known naturally occurring agonists of the PPAR pathway, which is proposed to be a primary mechanism via

Table 9.2 Summary of studies examining the effect of rTFA on CVD risk factors

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Randomized clinical trials				
Tricon et al. (2006)	Effect of dairy food enriched with CLA and VA on blood lipid profile, LDL atherogenicity, insulin resistance, and inflammatory markers.	Double-blind, crossover (6 weeks each), 32 healthy males, 34–60 years old. Daily dose: 1. 151 mg c9,t11-CLA + 312 mg VA 2. 1421 mg c9,t11-CLA + 4689 mg VA	↑ LDL:HDL ratio, no effect on inflammatory markers, insulin, glucose, triglycerides, total, LDL- or HDL-cholesterol, LDL particle size, or LDL oxidation.	VA/CLA-enriched dairy products do not appear to have a significant effect on blood lipid profile.
Raff et al. (2006)	Effect of CLA mixture and VA-enriched butter on arterial health	Double-blind, parallel 5-week intervention, 60 healthy young men. Daily dose: 1. 4.7 g CLA (c9,t11-:t10,c12-CLA = 1:1) 2. 3.6 g VA from butter 3. control diet low in VA and CLA	No effect of diets on blood pressure, isobaric arterial compliance, distensibility, or volume.	Milk fat high in either CLA or VA has no effect on arterial health in healthy young men.
Tholstrup et al. (2006)	Effect of butter high in VA and MUFA on risk markers of ischemic heart disease	Double-blind, 5-week parallel intervention, 42 healthy young men, 19–33 years old Daily dose: 0.5 g or 3.6 g of VA	VA diet had 6% ↓ total cholesterol and 9% ↓ HDL-cholesterol. No differences in TG, LDL, serum insulin, glucose, CRP, or urinary 8-iso-$PGF_{2\tilde{\alpha}}$.	Butter high in VA and MUFA reduces total and HDL cholesterol, but may be due to greater content of MUFA rather than VA per se.
Raff et al. (2008)	Effect of CLA mixture on risk markers of atherosclerosis, inflammation, and lipid peroxidation	Double-blind, parallel 5-week intervention, 38 healthy young men. Daily dose: 1. 0.3 g c9,t11-CLA 2. 2.5 g c9,t11-CLA + 2.1 g t10,c12-CLA	↑8-iso-$PGF_{2\alpha}$, no change in TG, TC, TC:HDL-C, CRP, insulin or glucose between groups	CLA mixture increases lipid peroxidation but has no effect on CVD risk compared to regular butter.
Sluijs et al. (2010)	Effect of CLA supplementation on markers of atherosclerosis and CVD risk factors in overweight and obese but otherwise healthy subjects	Double-blind, parallel 6-month intervention, $n = 401$. Daily dose: 1. Placebo (80% palm oil + 20% soybean oil, no CLA) 2. 2.5 g c9,t11-CLA + 0.6 g t10,c12-CLA	No effect of CLA on aortic stiffness, blood pressure, body composition, insulin resistance, or concentrations of lipids, glucose, CRP	This study does not support an anti-atherosclerotic effect of CLA mixture.

Continued

Table 9.2 *Continued*

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Sofi et al. (2009)	Effect of a sheep cheese enriched in VA and *c*9,*t*11-CLA on lipid parameters, inflammatory cytokines, and platelet function of healthy subjects	Crossover (10 weeks each), 6 females + 4 males, mean age = 45.6 years. 200 g/week of enriched and regular cheese. Daily dose: 1. 0.1 g VA + 0.05 g *c*9,*t*11-CLA 2. 0.9 g VA + 0.4 g *c*9,*t*11-CLA	↓IL-6, IL-8, and TNF-α, ↓extent of platelet aggregation induced by arachidonic acid.	Enriched dairy product causes favorable biochemical changes of atherosclerotic markers.
Risérus et al. (2004)	Effect of *c*9,*t*11-CLA on insulin sensitivity, blood composition, and lipid peroxidation in subjects with high CVD risk	Double-blind, parallel 3-month intervention, 25 abdominally obese men. Daily dose: 1. placebo (olive oil) 2. 2.5 g *c*9,*t*11-CLA + 0.2 g *t*10,*c*12-CLA	↓insulin sensitivity, ↑8-iso-$PGF_{2\alpha}$, no effect on body composition, plasma lipoprotein, TG or FFA.	CLA increases insulin resistance and lipid peroxidation in obese men.
Tholstrup et al. (2008)	Compare CLA mixture and *c*9,*t*11-CLA on CVD risk markers in postmenopausal women	Double-blind, parallel 16-week intervention, *n* = 75. Daily dose: 1. olive oil 2. 1.8 g *c*9,*t*11-CLA + 1.8 g *t*10,*c*12-CLA 3. 4 g *c*9,*t*11-CLA	↑TC : HDL-C, CRP, fibrinogen, PAL-1 in CLA mixture than *c*9,*t*11-CLA, ↑TG, and ↓HDL-Cl in CLA mixture than olive oil. Both CLA preparation increased lipid peroxidation.	CLA mixture has adverse effects on CVD markers whereas *c*9,*t*11-CLA is neutral except for a small increase in lipid peroxidation
Laso et al. (2007)	Effect of milk supplemented with CLA mixture on body composition and markers of metabolic syndrome	Double-blind, parallel 12-week intervention. 60 healthy men and women (BMI = 25–35 kg/m^2). Daily dose: 500 mL milk with or without 3 g CLA mixture	↓ total fat mass in overweight but not obese subjects in the CLA-milk group. No change in biochemical parameters of metabolic syndrome, hematological parameters, or renal function.	CLA mixture is not associated with adverse effect or biological change.
Animal feeding studies				
LeDoux et al. (2007)	Effect of individual CLA isomers on lipoprotein cholesterol in hamsters	Cholesterol-fed hamsters. 12 weeks. 1% w/w of *c*9,*t*11-CLA, *t*10,*c*12-CLA or CLA mixture	↓ total cholesterol, HDL, LDL, small dense LDL by *c*9,*t*11-CLA No significant changes in the *t*10,*c*12-CLA or the mixture group	*c*9,*t*11-CLA beneficially affects lipoprotein profile in this model. *T*10,*c*12-CLA and CLA mixture are less active.

Wilson et al. (2006)	Effect of *c*9,*t*11-CLA, *t*10,*c*12-CLA, and linoleic acid on plasma lipids and aortic cholesterol in hamsters	Cholesterol-fed hamsters. 12 weeks. 0.5% w/w *c*9,*t*11-CLA, *t*10,*c*12-CLA or LA	↓ plasma total cholesterol, HDL-C, non HDL-C, TG, cholesterol accumulation in aortic arch by *c*9,*t*11-CLA	C9,*t*11-CLA improves CVD risk factors.
Mitchell et al. (2005)	Effect of *c*9,*t*11- and *t*10,*c*12-CLA on atherogenesis in hamsters	High fat high cholesterol-fed hamsters. 12 weeks. 1% w/w *c*9,*t*11-CLA, *t*10,*c*12-CLA, LA	↓non HDL-C : HDL-C and aortic fatty streak lesion by *c*9,*t*11-CLA. No effect of *c*9,*t*11-CLA on plasma TG, LDL-C	Individual CLA isomers beneficially changes lipoprotein profile and reduces atherosclerotic lesion development, but not different than LA.
Arbones-Mainar et al. (2006)	Different atherogenic properties of *c*9,*t*11- and *t*10,*c*12-CLA in ApoE$^{-/-}$ mice	ApoE$^{-/-}$ mice were fed with 1% *c*9,*t*11-, *t*10,*c*12-CLA, or LA for 12 weeks	C9,*t*11-CLA ↓plasma cholesterol, free fatty acids, glucose, insulin, and aortic lesions, ↑apo A-I; *T*10,*c*12-CLA showed opposite effects.	C9,*t*11-CLA impedes atherosclerosis development while *t*10,*c*12-CLA is proatherogenic.
Toomey et al. (2003)	Effect of CLA on COX and PPAR-γ pathways in ApoE$^{-/-}$ mice	ApoE$^{-/-}$ mice were fed 1% *c*9,*t*11-CLA or COX-1 inhibitor for 16 weeks.	C9,*t*11-CLA retarded atherosclerotic lesion development and induced aortic lesion regression. ↑ PPAR- γ expression at aortic root	C9,*t*11-CLA benefits atherosclerosis as PPAR- γ agonist, not COX-1 inhibitor.
Kritchevsky et al. (2004)	Effect of individual *c*9,*t*11-CLA and *t*10,*c*12-CLA on the development and regression of cholesterol-induced atherosclerosis	Rabbits bearing atheromatous lesions were fed 0.5% *c*9,*t*11-, *t*10,*c*12-CLA, or mixture of 2 isomers for 90 days	↓ Atherosclerotic lesions in arch and thoracic area of aorta in *c*9,*t*11-CLA and mixture groups. *T*10,*c*12-CLA only ↓ lesions in aortic arch	CLA improves atherosclerotic lesions, and the efficacy is higher in high cholesterol diet.
Valeille et al. (2004)	Compare the efficacy of CLA and fish oil in modulating atherogenic risk markers	Cholesterol-fed hamsters were fed for 8 weeks with 1. control diet 2. 0.6% *c*9,*t*11-CLA 3. 1.2% CLA mixture 4. 1.2% CLA mixture + 1.2% fish oil 5. control diet + 1.2% fish oil	↑ LDL-receptor, scavenger receptor type B-1 by *c*9,*t*11-CLA	Part of the beneficial effects of CLA can be ascribed to *c*9,*t*11-isomer and are boosted by fish oil.

Continued

Table 9.2 *Continued*

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Meijer et al. (2001)	Differential impact of 2 TFA, vaccenic acid and elaidic acid (EA), on lipid and eicosanoid metabolism	Cholesterol-fed hamsters were fed for 5 weeks: 1. MCFA (8:0 + 10:0) 2. SFA (16:0) 3. MUFA (*c*9-18:1) 4. EA 5. VA	↓LDL and TG content compared to SFA No difference in LDL and VLDL cholesterol or TG, total cholesterol, or free cholesterol compared to MUFA, EA, or MCFA. ↑LDL/HDL ratio compared to EA	Both EA and VA lowers CVD risk factors, but this study does not support that EA is more detrimental than VA.
Bassett et al. (2010)	Compare the effect of VA and EA on atherosclerosis risk factors	$LDLr^{-/-}$ mice were fed one of eight experimental diets for 14 weeks: 1. regular fat 2. elaidic shortening 3. regular butter 4. VA butter 5. 2% cholesterol 6. 2% cholesterol + elaidic shortening 7. 2% cholesterol + regular butter 8. 2% cholesterol + VA butter	VA butter ↓ serum cholesterol and TG levels compared to EA and regular butter diet. VA butter is more effective when the diet contains higher cholesterol.	EA shortening diet was atherogenic while VA butter diet was not atherogenic.
Bauchart et al. (2007) Roy (2007)	Impact of natural (milk) CLA, VA, and *t*10-18:1 on the risk of atherogenesis	Male New Zealand white rabbits fed one of three diets for 12 weeks: 1. *T*10-18:1 2. VA + CLA (0.8% VA + 0.3% CLA) 3. Low in all three fatty acids	Compared to t10-18:1 diet, VA + CLA butter↓ VLDL, plasma total cholesterol, phospholipids, and apolipoprotein B. ↓ aortic lipid infiltration vs. *t*10-C18:1, but not control diet. ↑ liver TG vs. other diet groups.	VA/CLA butter has neutral effect toward risk of atherogenesis, whereas *t*10-18:1 is detrimental.
Tyburczy et al. (2009)	Effect of EA and VA on CHD risk in hamsters	Cholesterol-fed hamsters were fed either a control "Western" diet or 2% supplementation of PHVO, VA, or EA for 4 weeks.	VA and EA reduced plasma total:HDL-C and non HDL-C:HDL-C ratios while PHVO increased these values.	Hypercholesterolemic effect of PHVO are not dependent on EA or VA.

Wang et al. (2008) Blewett et al. (2009)	Effect of VA on CVD risk in rats with dyslipidemia and the metabolic syndrome	Lean and obese JCR : LA-cp rats were fed either a control "Western" diet or 1% VA for 3 weeks.	↓ plasma TG, no adverse effect on body weight, plasma glucose/insulin, total or LDL-cholesterol ↓IL-2, TNF-α production by splenocytes	VA has neutral effect under normal condition and reduces circulating TG under dyslipidemic conditions.
Wang et al. (2009) Ruth et al. (2009)	Effect of VA on CVD risk in rats with dyslipidemia and the metabolic syndrome	Obese JCR : LA-cp rats were fed either a control "Western" diet or 1% VA for 16 weeks.	↓ plasma TG, total and LDL-cholesterol, free fatty acid, haptoglobin, intestinal remnant lipoprotein, hepatic fatty acid synthesis ↓proinflammatory cytokine production by splenocytes	VA has reduced CVD risks and improves inflammatory response under conditions of dyslipidemia and the metabolic syndrome.
Valeille et al. (2005)	Compare the efficiency of CLA and fish oil in modulating atherogenic risks	Cholesterol-fed hamsters were fed for 12 weeks with 1. 20% butter fat (1% VA + 0.4% *c*9,*t*11-CLA (B diet) 2. 20% butter fat + 1% *c*9,*t*11-CLA (BR diet) 3. 20% butter fat + 1% fish oil (BF diet)	BR diet had lowest aortic lipid deposition, reduced plasma non-HDL:HDL ratio, improved antioxidized LDL paraoxonase activity, downregulated expression of inflammatory-related genes (TNF-α, IL-1β, COX-2)	Milk fat rich in *c*9,*t*11-CLA reduces atherogenic process in hyperlipidemic hamsters.
Lock et al. (2005)	Effect of VA/CLA enriched butter on plasma lipoprotein and tissue fatty acid profile	Cholesterol-fed hamsters were given the following three diets for 4 weeks 1. 20% standard butter 2. 5% standard butter + 15% VA/CLA butter 3. 15% standard butter + 5% PHVO	VA/CLA butter ↓ plasma cholesterol, VLDL-C, (VLDL + LDL):HDL ratio	VA/CLA butter improves plasma lipoprotein profile and reduces risk of atherosclerosis.
Cell culture studies				
Ringseis et al. (2008)	Effect of CLA on macrophage cholesterol accumulation as PPAR-α, γ agonists	Mouse macrophage-derived foam cells RAW264.7 were treated with *c*9,*t*11-, *t*10,*c*12-CLA, or LA at 50μM for 24 hours.	Both CLA isomers reduced cholesterol accumulation, stimulated cholesterol efflux, and upregulated CD36, ABCA1, NPC-1, NPC-2, and LXR-α.	CLA reduced macrophage cholesterol accumulation via PPAR-dependent pathways.
Stachowska et al. (2009)	Effect of CLA isomers on COX-1 and COX-2 activity of macrophages	Macrophages were treated with *c*9,*t*11-, *t*10,*c*12-CLA, or LA at 30μM for 48 hours	Both CLA isomers reduced TXB_2, PGE_2 via inhibiting COX-1 and COX-2 activity.	CLA inhibits COX activities via competing with arachidonic acid.

which CLA elicits pleiotropic effects (Banni, 2002; Brown et al., 2003). Upregulation of PPAR-γ has been shown to normalize insulin sensitivity, improve lipid metabolism and the clearance of lipoproteins, as well as restore vascular contractility and endothelial function (Biscetti et al., 2009). Immunohistochemical staining of aortic vessels have shown an increased expression of PPAR-γ in *c*9,*t*11-CLA supplemented animals compared to controls (Toomey et al., 2003). Collectively, these changes may partially explain the reduced accumulation of cholesterol in arterial vessels and attenuated progression of atherosclerosis. Moreover, treatment with *c*9,*t*11-CLA *in vitro* resulted in augmented acceptor-dependent cholesterol efflux from macrophage-derived foam cells and increased mRNA expression of genes involved including CD36, ABCA1, LXR-α, NPC1, and NPC2 (Ringseis et al., 2008). CLA has also been shown to suppress the production of proinflammatory cytokines(such as PGE_2 and TNFα), as well as IFN-γ-induced COX-2 activity in mouse and human macrophages, both of which are considered characteristic changes of a PPAR-agonist treatment (Iwakiri et al., 2002; Yu et al., 2002; Stachowska et al., 2009).

In addition to the modulation of PPAR-γ expression, CLA has also been reported to increase the expression of the hepatic lipoprotein receptors (apoB100/E-receptor, more commonly known as the "LDL"-receptor) responsible for the clearance of cholesterol-rich lipoproteins, including LDL and remnant lipoproteins (Dane-Stewart et al., 2003; Valeille et al., 2004) Upregulation of the hepatic apoB100/E receptor and the inhibition of HMG-CoA reductase are the primary mechanisms targeted by lipid lowering therapies, such as the statin compounds (Dane-Stewart et al., 2003). Valeille and colleagues recently reported that an increased mass of LDL-receptor in the liver of hamsters supplemented with either the single *c*9,*t*11 isomer or mixed isomeric CLA preparations (Valeille et al., 2004). However, the increase in LDL-receptor mass was independent of the effects on HMG-CoA reductase in reducing cholesterol synthesis, suggesting that CLA may have direct regulatory effects on the molecular signaling/production pathways of LDL-receptor expression (such as the insulin signaling cascade involving PI3-kinase and/or SREBP-1 pathway).

9.6 VACCENIC ACID

*T*11-vaccenic acid is the predominant *trans* monoene in ruminant fats (50–80% of total *trans* fat) (Lock et al., 2004). As mentioned earlier, VA is produced via the incomplete biohydrogenation of PUFA (e.g., linoleic and linolenic acid) by microorganisms in the rumen (Lock et al., 2004). It is also interesting that dietary VA can be desaturated to *c*9,*t*11-CLA in ruminants, rodents, and humans (Santora et al., 2000; Turpeinen et al., 2002). There has been little research attention to this natural *trans* fat, despite the fact that it is the precursor for *c*9,*t*11-CLA, which is considered beneficial to cancer and CHD prevention. More recently, agricultural scientists have made efforts to increase the *c*9,*t*11-CLA content of animal fats, which has resulted in an elevated VA production (Fievez et al., 2003; Lynch et al., 2005). Hydrogenated plant oils are another rich natural source of VA, and it has been recently estimated that plants may contribute to about 13–17% of total VA intake (Wolff et al., 2000). The presence of VA in industrial *trans* fats has raised the question as to whether VA elicits the same adverse health effects as industrially produced *trans* fats. However, the bioactivity of VA per se and how it could impact chronic disease remain unclear. Due to the lack of evidence, VA remains part of the *trans* fat content on food labels, while CLA has been exempted in several countries.

9.6.1 VA modulates carcinogenesis

There has been limited evidence associating VA with the suppression of cancer development (summarized in Table 9.1). The majority of studies (albeit limited) using cancerous cell lines or rodent tumor models have demonstrated that VA can reduce cell growth and/or tumor metabolism (Banni et al., 2001; Lock et al., 2004; Lampen et al., 2005). The treatment VA on HT-29 colon cancer cell line showed obvious inhibitory effect on tumor growth, while elaidic acid (EA, *t*9 C18:1, a major TFA found in PHVO) had no effect (Awad et al., 1995). Another study using a mammary cancer cell line, MCF-7, and a colonic cancer cell line, SW480, demonstrated reduced cell growth in both cell types after a 4-day treatment with 20 μg/mL pure VA (Miller et al., 2003). Moreover, the SW480 cell line had enhanced DNA fragmentation and depleted cytosolic glutathione (GSH) leading to cell death. Further, the eicosanoid profile incorporated into phospholipids in SW450 cells was altered with VA treatment (similar to that observed during *c*9,*t*11-CLA treatment), suggesting VA may share its metabolic fate with *c*9,*t*11-CLA (Miller et al., 2003). However, another study claimed that VA per se did not affect inflammatory markers in Caco-2 cell line, but rather played the role of the precursor to endogenous synthesis of *c*9,*t*11-CLA (Reynolds et al., 2008). Animal studies demonstrating the association between VA and carcinogenesis have been rather sparse, largely due to the prohibitive cost of pure VA oil. Sauer et al. has performed *in-situ* perfusion on a mouse hepatoma model with purified VA for 2.5 hours and found that fatty acid uptake by the tumor was significantly inhibited together with decreased cAMP content and extracellular signal-regulated kinase p44/p42 phosphorylation (Sauer et al., 2004), supporting the idea of an acute inhibitory effect of VA on tumor growth. In a short-term feeding study, Banni and colleagues fed MNU-injected female rats with 2% VA-supplemented basal diet. After 6 weeks, treated rats had almost 50% less premalignant lesions compared to control rats (Banni et al., 2001).

Overall, evidence collected from both animal and *in vitro* studies to date appear to be convincing in regard to the anticancer properties of VA. The mechanisms remain elusive, but could be in part due to the *in vivo* conversion of VA to *c*9,*t*11-CLA (Banni et al., 2001; Corl et al., 2003, Lock and Bauman, 2004). However, several additional mechanisms have been proposed, including changes in phosphatidylinositol hydrolysis, reduced proliferation, inhibition of fatty acid uptake, and increased apoptosis (Awad et al., 1995; Miller et al., 2003; Sauer et al., 2004).

9.6.2 VA modulates CVD risk factors

At present, there are relatively few research groups who have explored the effect of purified VA preparations on CVD risk factors due largely to its limited availability. A number of years ago, Meijer et al. fed hamsters a diet with VA, EA, oleic acid, palmitic acid, or a combination of medium-chain saturated fatty acid (SFA) for 4 weeks (10% of energy). The effect of VA on blood cholesterol profile and two lipid metabolizing enzymes (cholesteryl ester transfer protein [CETP] and phospholipid transfer protein [PLTP]) did not differ from oleic acid but were lower than saturated fat (Meijer et al., 2001). More recently, a 2.0% w/w supplementation of purified VA, EA, and PHVO were compared to a control "Western" diet in the hamster model to assess their impact on CHD risk factors (Tyburczy et al., 2009). After 4 weeks of treatment, PHVO increased the total : high-density lipoprotein (HDL)-cholesterol ratio while VA and EA decreased the ratio compared to controls. Plasma TG was not affected by any diet. The findings imply the possibility that there may

be other bioactive components in PHVO, other than EA or VA, that are responsible for the detrimental hypercholesterolemic effects. In contrast to the diet-induced hypercholesterolemic hamster model, our own research group used a unique disease model, the JCR:LA-*cp* rat, which spontaneously develop symptoms of the metabolic syndrome, dyslipidemia, prediabetes, as well as cardiovascular complications. We have reported a distinct hypo-triglyceridemic property of purified VA following a 3-week supplementation without adverse effect on body weight or insulin metabolism (Wang et al., 2008), accompanied by improved proinflammatory markers (interleukin [IL]-2 and tumor necrosis factor [TNF]-α) (Blewett et al., 2009). Interestingly, VA showed no effect in these parameters in normal rats, suggesting that the efficacy of VA to benefit CVD might be more profound under disease conditions. When rats were fed similar amount of VA for an extended period (16 weeks), plasma lipid profile showed a consistent and substantial improvement in circulating concentrations of TG, total cholesterol, LDL-cholesterol, haptoglobin, as well as intestinal remnant lipoprotein metabolism (Wang et al., 2009). In addition, the effect of VA on the inflammatory response (e.g., splenocyte cytokine production) remained consistent with the previous acute-feeding study (Ruth et al., 2009). These findings indicate that feeding VA may have profound impact on improving inflammatory responses and lipid parameters, especially TG, associated with the development of CVD, and the protective effect would be secured with prolonged feeding period. Unfortunately, at the time of writing, there has been no clinical study published so far using purified VA as the primary intervention, leaving the effect of this rTFA in human population to be revealed.

Although limited, animal feeding trials in rodent models with either induced or spontaneous dyslipidemia do not support adverse health effect of VA on markers of CHD risk. In contrast, it has been proposed that VA supplementation may improve dyslipidemia, by lowering circulating TG and/or cholesterol, thus attenuating atherosclerotic progression. The hypolipidemic benefits of VA have been reported to occur independently to the *in vivo* conversion to CLA. Further, it has been suggested that VA may modulate eicosanoid production to regulate a variety of downstream metabolic pathways involved in atherogenesis, such as lipid metabolism, immune response, vascular function, as well as platelet aggregation (Banni, 2002).

9.7 DAIRY FAT ENRICHED WITH VA AND CLA

Considering the limited availability of purified rTFA isomers, many research groups have chosen to enrich rTFA in dairy fat, which bears several advantages not only in cost but also in the synergistic bioactivity of both VA and *c*9,*t*11-CLA.

9.7.1 Enriched dairy fat modulates carcinogenesis

Rats with MNU-induced tumor appeared to develop less mammary tumors when fed butter enriched with VA and *c*9,*t*11-CLA. Premalignant lesions, tumor growth, and cell proliferation were also significantly reduced in a dose-dependent manner (summarized in Table 9.1). In MCF-7 mammary cancer cell culture, both CLA-containing milk fat and *c*9,*t*11-CLA isomer suppressed cell growth and increased lipid peroxidation in a dose-dependent manner (O'Shea et al., 2000). The consistent results, while still limited, indicate that tumor development appears to be reduced by VA/CLA-enriched dairy fat.

9.7.2 Enriched dairy fat modulates CVD risk factors

A majority of the studies that have explored the bioactivity of enriched dairy fat on CVD risk indices have been generated from rodent trials (summarized in Table 9.2). Valeille et al. reported that feeding 20% butter fat led to increased reverse cholesterol transport potential, decreased aortic cholesterol-ester deposition, LDL-peroxidability index, as well as IL-1 mRNA abundance in hamster aorta (Valeille et al., 2005). Bassett et al. found that $LDLr^{-/-}$ mice consuming *trans* fatty acids sourced from EA-rich hydrogenated vegetable shortening for 14 weeks had greater atherosclerosis than a butter enriched in VA (Bassett et al., 2010). In addition, when a VA/CLA-enriched butter was fed to rodents, serum cholesterol, TG, and the extent of atherosclerosis were reduced compared to regular butter (Lock et al., 2005; Bassett et al., 2010). Two studies using a rabbit model have compared the effects of a VA/CLA-enriched dairy diet to either a *t*10-C18:1 (another major TFA in PHVO) diet or a control diet low in these fatty acids. These studies reported a neutral effect of VA/CLA butter on risk factors of atherogenesis as well as less aorta fatty streak development (Bauchart et al., 2007). However, the studies in rabbits also reported that the ratio of atherogenic lipoproteins [very low-density lipoproteins (VLDL) + LDL] and antiatherogenic HDLs was significantly lower in the butter group compared to the *t*10 C18:1 group (Roy, 2007). When VA/CLA-enriched dairy products were given to several groups of healthy volunteers (summarized in Table 9.2), the lipid-lowering benefits appeared to be diminished, possibly due to relatively short intervention periods or, similar to previously discussed CLA trials, targeting populations being relatively healthy.

9.8 DISCUSSION

Recent research on ruminant TFA has demonstrated a protective effect against the development of several types of cancer as well as CVD, which is distinctive from the detrimental health outcomes from iTFA. We speculate that the inconsistency between experimental models and in pilot human studies could possibly be due to undefined dosage, duration, and the targeting background (i.e., healthy vs. diseased conditions) of the dietary rTFA intervention. Meanwhile, we propose that enrichment of dairy products with VA and CLA would synergistically enhance their bioactivity and protective benefits. As cumulative evidence appears to support a protective role of rTFA, it is important for public perspective to be aware of these changes; unfortunately, in the latest WHO report on *trans* fat, rTFA was implied to have similar detrimental effect on CHD risk without acknowledging the recent findings of their protective properties (Uauy et al., 2009).

On the other hand, the current public perception of dairy fat continues to be negative due to dietary recommendations that encourage low-fat dairy options and education that saturated fats from animal products are associated with higher risks of developing CVD. In fact, dairy food only contributes to a small part (less than 15%) of total dietary SFA intake, and is thus not necessarily responsible for the prevalence of CVD per se. The skimming process required for manufacturing low-fat or fat-free dairy product has concomitantly sacrificed other beneficial components associated with dairy fat (e.g., fat-soluble vitamins), not only in their absolute abundance, but also in their bioavailability. It is also interesting to note that while CLA supplements are available widely in the market, the bioavailability of CLA to the human body tends to be higher from natural food sources

(Burdge et al., 2004). On the other hand, being one of the major components in the Western diet, dairy foods remain a natural means to enhance the quality of dietary fats.

We also appreciate that there are ongoing unresolved issues that may hinder the application of these findings to industry. First, the level and duration of consumption of rTFA from natural sources that will provide a so-called "health" effect are still unclear. Most animal and cell culture studies exhibiting a beneficial effect of rTFA typically used super-physiological dose (3–5% of energy) considered not achievable in a typical Western diet (less than 0.5% of energy) without supplementation. Very few clinical trials to date have demonstrated convincing outcomes using enriched dairy product. The etiology of different chronic disease is complicated and multifactorial and confounds ongoing interpretation. Second, finding a cost-efficient method to enhance rTFA production could become vital for promoting this strategy nationwide (and/or globally). Meanwhile, rTFA is naturally present in the form of TG and phospholipids, whereas in most of the studies, it has been fed as single isomers. To what extent this compositional difference would influence metabolism in the intestine and liver has not been comprehensively assessed. Lastly, the ability to enrich rTFA in ruminant fat is limited and the extent to which the enrichment process can be challenged is unclear. Northern based dairy and beef production remain restricted by the lack of grass finishing periods relative to central and southern production sites. This in turn places great pressure on Northern sites to supplement their feed regimens in order to complete for an enriched VA/CLA (rTFA) healthy fatty acid profile.

The development of fortified dairy products with levels of rTFA equivalent to animal studies has become one of the obstructing factors hindering research progress. Indeed the application of genomic and bioengineering technology could potentially assist with strain screening and selective breeding of those with higher productivity. Meanwhile, it is essential to expand our understanding of the beneficial health effects of rTFA. Continued experimental research should be devoted to clarifying the effects of rTFA with a focus on study protocols, including the appropriateness of animal models (particularly in the disease setting), duration and dosage of rTFA in humans, as well as the differential effects of the sources of *trans* fat. Acknowledging the challenges ahead, the novel findings of the protective benefits of rTFA against CVD and several types of cancer have provided excellent opportunities that could subsequently benefit the nutrition health of the population.

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10 Nanotechnology for Cerebral Delivery of Nutraceuticals for the Treatment of Neurodegenerative Diseases

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10.1 INTRODUCTION

A century after its first description, Alzheimer's disease (AD) represents the most common form of dementia. AD is characterized by slow, chronic, and progressive neurodegeneration, which results in cognitive impairment and functional disturbances. In the brain, AD is characterized by amyloid-β peptide. (Aβ) deposits, neurofibrillary tangles (NFTs), synapse loss, and extensive oxidative stress. Aβ-induced oxidative stress is expressed as protein oxidation, lipid peroxidation, free radical formation, DNA oxidation, and neuronal cell death. The prevalence and incidence of this neuronal disorder increase with age. It is estimated that more than 24 million people suffer from dementia today, with 4.6 million new cases every year; the number of people affected will double every 20 years, reaching 81 million by 2040 (Ferri et al., 2005). Therefore, there is a critical need to develop new strategies to prevent or to slow down the progression of AD. The prevalence of AD is strongly influenced by diet and nutrition. Recent studies suggested that certain diets including beverages, polyphenols, functional foods, and nutraceutical compounds could be associated with a lower incidence of AD. However, once absorbed, these molecules are subjected to modifications and their concentrations are usually very low in plasma.

Over the last decade, nanotechnology has been explored extensively as an area of potential research for the development of newer therapeutic modalities for the treatment of neurological disorders such as AD. The enhanced bioavailability and/or efficacy associated with the formulation of targeted nanoparticles (NPs) of various drugs and bioactive agents used in neurodegenerative disorders may provide a possible solution to overcome many of the challenges for the treatment of these diseases. This chapter will focus on the most important materials to produce NPs for cerebral delivery of nutraceutical compounds delivered orally. Enhanced bioavailability of these compounds in the future will bring them to the forefront of therapeutic agents for the prevention and treatment of AD.

AD is the most common neurodegenerative disorder related to aging. AD is characterized by relatively slow chronic but progressive neurodegeneration and impairment in cognition, behavior, and functionality. The disease is both multifactorial and heterogeneous,

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involving aberrant protein processing characterized by the presence of both intraneuronal protein clusters composed of paired helical filaments of hyperphosphorylated tau protein (NFTs), extracellular protein aggregates (senile plaques [SPs]), and loss of synaptic connections within selective brain regions (Selkoe, 2001). A main component of amyloid plaques is the amyloid-β peptide. (Aβ), generated by the proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. Aβ exists in many forms, such as soluble, aggregated, oligomeric, protofibrillar, and fibrillar (Dahlgren et al., 2002; Caughey and Lansbury, 2003). Interestingly, cumulative research demonstrates that the oligomeric form of Aβ is highly toxic (Walsh et al., 2001; Drake et al., 2003; Fawzi et al., 2007).

The causes of dementia are actually unknown, but some epidemiological studies support the hypothesis that lifestyle-related factors are associated with the development of dementia in late life. The vascular and related factors that have been associated with dementia and cognitive decline include hypertension and elevated blood pressure (Kester et al., 2010), total cholesterol (Martins et al., 2009), diabetes mellitus (de la Monte et al., 2009), body mass index (Chen et al., 2010), and the metabolic syndrome (Forti et al., 2010). In contrast, physical exercise (Rolland et al., 2010) and education (Paradise et al., 2009) appear to have clear protective effects on cognitive functioning with possible reductions in dementia incidence.

Some findings showed that older African Americans and Japanese living in the United States have a much higher prevalence of AD (6.24% and 4.1%, respectively) than those still living in their ethnic homelands (<2%), thereby suggesting that the prevalence of AD is more strongly influenced by diet and nutrition, environment, and/or lifestyle than by genetics (Gillette Guyonnet et al., 2007).

Due to the increasing longevity of the population, a dramatic rise in AD prevalence is expected (Hirtz et al., 2007). Current available pharmacological treatments reduce symptoms of AD rather than prevent an ultimate decline in cognitive and behavioral functioning. Those compounds are costly and are often associated with many adverse effects including confusion, nausea, diarrhea, weight loss, vomiting, dizziness, fatigue, and headache. Thus, effective strategies for early prevention or delayed onset of AD should identify risk-reducing modifiable environmental/lifestyle factors such as diet. Recent findings demonstrate that intake of nutraceutical compounds or a healthy diet may have a positive impact on possible risk factors for cognitive decline. However, the concentrations of most of nutraceutical compounds or diet components, for instance, polyphenols, are usually very low in plasma after their intake at the nutritional dose because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. Additionally, once absorbed, some of them are subjected to three main types of conjugation: methylation, sulfation, and glucuronidation.

Recent progress had focused on improving drug delivery for neurodegenerative diseases using nanomedicines. NP-loaded bioactive compounds is an active area of research whose aim is to increase the oral bioavailability of poorly absorbed compounds. In this chapter, we will discuss the effectiveness of controlled release and delivery of nutraceutical or functional food ingredients for the treatment and prevention of AD.

10.2 OXIDATIVE STRESS IN MILD COGNITIVE IMPAIRMENT (MCI) AND AD

The brain is subject to oxidative damage because it contains high levels of polyunsaturated fatty acids (PUFAs), high levels of redox transition metal ions, and a high oxygen consump-

tion. In contrast, the levels of antioxidants are relatively low compared with other organs. Among different pathogenic hypotheses, the oxidative stress hypothesis in AD is attractive because it associates several others such as the trace element hypothesis (iron, copper) (Smith et al., 1997; Sayre et al., 1999), the mitochondrial hypothesis (Aliev et al., 2009; Rhein et al., 2009), and the β-amyloid peptide (Aβ) hypothesis (Hardy and Selkoe, 2002; Hardy, 2009). In recent years, numerous studies have strongly suggested that free radical-mediated oxidative damage plays an early role in the pathogenesis of AD (see review by Christen, 2000; Sonnen et al., 2008). In mild cognitive impairment (MCI) and early AD, multiple articles have documented increased lipid peroxidation, protein carbonylation, glycol-oxidation, and DNA and RNA oxidation with widespread oxidative damage throughout multiple brain regions (Markesbery et al., 2005). Interestingly, increased levels of oxidative markers in a disease-dependent manner are correlated with the Mini-Mental Status Examination scores (Ansari and Scheff, 2010).

PUFA and their metabolites contribute to some crucial physiological roles, including membrane structure and fluidity, cell signaling, energy production, and regulation of gene expression. PUFA, especially arachidonic and docosahexenoic acid, which build the brain phospholipids, are vulnerable to reactive oxygen species due to their double bonds. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom near the double bond and thus facilitates $H^{\bullet}$ abstraction from a methylene group ($-CH_2-$). This reaction generates an unpaired electron on the carbon ($-{}^{\bullet}CH-$). An important aspect of lipid peroxidation is its self-propagating process, which proceeds until the substrate is consumed or termination occurs. In this way, lipid peroxidation is fundamentally different from other forms of free radical injury in that it is a self-sustaining process capable of extensive tissue damage. There are two outcomes to lipid peroxidation: structural damage to membranes and generation of oxidized products, some of which are chemically reactive and can covalently modify macromolecules; others may be considered as second toxic messengers that disseminate and augment initial free radical events. Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport, and inhibition of metabolic processes. Lipid peroxidation releases high amounts of different by-products such as isoprostanes (IsoPs), neuroprotanes, malondialdehyde (MDA), bioactive α,β-unsaturated aldehydes including 4-hydroxy-2-*trans*-nonenal (HNE), and acrolein. There has been much focus on the possible role of lipid peroxidation-derived aldehydes in contributing to neuronal dysfunction in neurodegenerative diseases associated with oxidative stress. Numerous studies have documented increased levels of reactive products of lipid peroxidation in diseased regions of the AD brain (Montine et al., 1997, 1998; Calingasan et al., 1999; Ramassamy et al., 1999, 2000; Casadesus et al., 2007; Perluigi et al., 2009; Reed et al., 2009; Sultana, 2009; Singh et al., 2010). Also, proteins modified by lipid peroxidation products were present in diseased regions of the brain but not in regions uninvolved in AD. Within diseased regions of the brain, most studies have observed immunoreactivity of by-products of lipid peroxidation with neuronal cytoplasm and NFTs (Montine et al., 1997, 1998; Markesbery and Lovell, 1998). In addition, these reactive products of lipid peroxidation have been proposed to participate in pathologic posttranslational modifications of the paired helical filaments formed by tau and Aβ peptide. *In vivo* studies showed that increased lipid peroxidation leads to upregulation of BACE1 expression, which may lead to increased Aβ(1–42) production (Tamagno et al., 2005). Lipid peroxidation products and Aβ(1–42) have been shown to induce c-Jun N-terminal kinase (JNK) pathways, leading to neuronal apoptosis (Tang et al., 2008). There is growing evidence that oxidative stress and by-products of lipid

peroxidation can produce neurotoxicity by causing nerve terminal dysfunction and synaptic loss. These observations are of great importance because lipid peroxidation is not only a consequence of AD amyloidosis (Pratico et al., 2001; Bradley et al., 2010) but also strengthen the notion that brain oxidative stress and lipid peroxidation are potential therapeutic target early in the course of AD.

Protein carbonyls and 3-nitrotyrosine, which are the markers of protein oxidation are also elevated in AD (Sultana et al., 2006a,b). Protein carbonyls are present in both tangles- and non-tangles-bearing neurons in frontal lobe or hippocampus, both brain area involved in cognitive and memory processing. Nitrotyrosine and dityrosine cross-linked proteins are elevated eightfold and threefold, respectively in the hippocampus and neocortical regions of AD brain as compared to age-matched controls (see review by Sonnen et al., 2008). By using redox proteomics, specific elevation of oxidatively modified proteins have been identified in the hippocampus and the parietal lobe of the AD brain, such as α-enolase, heat shock cognate 71 (HSC 71), creatine kinase BB (CK BB), glutamine synthase (GS), and ubiquitin carboxy-terminal hydrolase L-1 (UCHL-1) (Castegna et al., 2004; Sultana et al., 2010).

Higher levels of multiple oxidized bases from nuclear and mitochondrial DNA in AD brain are found in frontal, parietal, and temporal lobes as compared to control brain regions. Moreover, mitochondrial DNA had approximately 10-fold higher levels of oxidized bases than nuclear DNA. These data are consistent with higher levels of oxidative stress in mitochondria. DNA from temporal lobe showed the most oxidative damage, whereas cerebellum was only slightly affected in AD brains. These results suggest that oxidative damage to mitochondrial DNA may contribute to the neurodegeneration observed in AD. DNA repair mechanisms have a critical role in protecting the genome. Several studies have shown a decline in repair of 8-OHdG in AD (Moreira et al., 2008). RNA oxidation is also an important event in the pathogenesis of AD as up to 50% of mRNAs purified from AD frontal cortices were oxidized and RNA oxidation occurred to a large extent in those neurons that are especially vulnerable to degeneration in AD (Nunomura et al., 2009).

10.3 EFFICACY OF SELECTED COMPONENTS OF NUTRACEUTICAL COMPOUNDS IN THE AMYLOID CASCADE AND IN THE PREVENTION OF AD

The "amyloid hypothesis" was first introduced in 1992 to explain the pathophysiology of AD (Hardy and Higgins, 1992). According to this hypothesis, abnormal proteolytic cleavage of APP by β- and γ-secretases leads to excessive extracellular accumulation of Aβ in the AD brain (Selkoe, 1996) (Fig. 10.1). Aβ exerts neurotoxic and synaptotoxic affects both *in vitro* and *in vivo*. Pathologically accumulated Aβ might directly interact with neuronal membranes or indirectly stimulate diverse intracellular signaling pathways to impair neuronal synapses and dendrites, and cause local oxidative stress reactions and sustained inflammatory responses. Through these various and multiple mechanisms, Aβ accumulation leads to the progressive loss of neurons, disintegration of neural circuits, and neurological decline characteristic of AD. Additionally, soluble Aβ monomers assume a random coil or β-helix conformation; however, in AD they undergo a structural change into a pleated β-sheet. These structural changes induce low-molecular-weight oligomers,

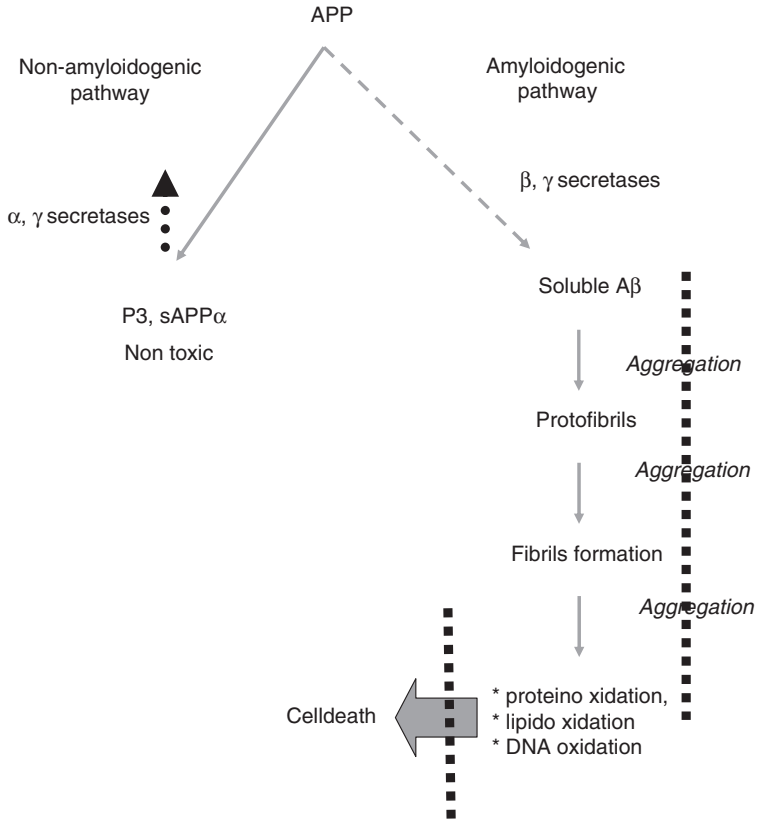

Figure 10.1. APP cascade and potential targets of nutraceutical compounds. Dotted lines indicate targets of nutraceutics.

higher molecular weight complexes (protofibrils and Aβ-derived diffusible ligands or ADDLs), mature fibrils and amyloid plaques in the neuropil and the vasculature (Walsh et al., 1999).

For many years, it was believed that the toxic effects of Aβ were a result of the mature Aβ fibrils; however, recent studies suggest that low-molecular weight, soluble, oligomeric forms of Aβ(1–42) rather than Aβ(1–40) (Lambert et al., 1998) are more neurotoxic than the mature Aβ fibrils (Hartley et al., 1999). Accumulation of high-molecular-weight (HMW) soluble oligomeric Aβ species is hypothesized to initiate a cascade of cellular events resulting in synaptic failure, neuronal injury, apoptotic neuronal death, and ultimately, cognitive and functional decline including deficits in spatial memory (Cleary et al., 1995, 2005; Jacobsen et al., 2006). Therefore, strategies to inhibit Aβ oligomer formation as well as Aβ fibrils would represent an interesting approach for the prevention or treatment of AD (Necula et al., 2007). Rational design of peptide inhibitors directed against key residues important for aggregation and stabilization of fibrils has demonstrated effectiveness at inhibiting fibrillogenesis. To date, it has been reported that various compounds inhibit the formation and extension of Aβ fibrils, as well as destabilizing Aβ fibrils *in vitro*. The first anti-amyloid drug to reach a pivotal clinical trial was tramiprosate (Greenberg et al., 2006). Tramiprosate is a glycosaminoglycan mimetic that binds to monomeric Aβ, thereby

reducing aggregation and neurotoxicity while promoting clearance from brain. However, this compound failed to demonstrate a beneficial effect on the primary outcomes, change in cognition, and clinical stage for the North American Phase III study which included 1052 patients with mild to moderate AD. This compound is now marketed as a nutraceutical for memory protection (Swanoski, 2009). Recently, a long-term administration of a polyphenol rich grape seed extract (GSPE), namely Meganatural-AZ®, has demonstrated the ability to exert potent anti-Aβ-oligomerization activity (Ono et al., 2008) and to attenuate the oligomerization of Aβ peptides into HMW soluble species in the brain of a mouse model of AD (Wang et al., 2008). This bioactive GSPE preparation is composed of proanthocyanidins (PACs), which is the most abundant and complex class of polyphenol found in grapes. Acute and repeated dose studies have demonstrated that bioavailability of GSPE phenolics including gallic acid and PAC's monomers such as catechins is poor (<2% of oral dose) with intestinal absorption of PAC dimers, timers, and complex oligomers observed to be extremely limited (Konishi et al., 2004; Tsang et al., 2005). No detectable amounts of free gallic acid, PAC's or their methylated metabolites were found in brain extracts of rats acutely treated with 50, 100, or 150 mg GSPE/kg body weight. However, detectable levels of free catechins were observed in brain tissues (Ferruzzi et al., 2009). Additionally, small-molecule inhibitors, including polyphenolic compounds such as GSPE, wine-related polyphenols (resveratrol, myricetin, morin, and tannic acid), curcumin, ferulic acid, rosmarinic acid, and (−)-epigallocatechin gallate (EGCG), had displayed an anti-Aβ aggregation effects *in vitro* through distinct mechanisms. Interestingly, some of them have demonstrated effectiveness at reducing Aβ levels when administered to transgenic mouse models of AD (Bieschke et al., 2010; Lim et al., 2001; Ueda et al., 2002; Ono et al., 2002a,b, 2004a,b, 2005a,b, 2006a,b,c,d, 2008; Hirohata et al., 2005, 2007; Marambaud et al., 2005; Rezai-Zadeh et al., 2005; Yang et al., 2005; Hamaguchi et al., 2006, 2009; Garcia-Alloza et al., 2007; Morinaga et al., 2007; Riviere et al., 2007; Ehrnhoefer et al., 2008; Inbar et al., 2008; Shoval et al., 2008). Among the polyphenolic compounds with emerging interest are curcumin, resveratrol, and catechins. These results are corroborated by some epidemiological studies that have suggested a positive relationship between consumption of these compounds and the prevention of AD. For instance, in the Swedish Twin Registry study, among the 3779 members who completed a diet questionnaire approximately 30 years before cognitive screening and full clinical evaluation for dementia, a medium or great intake of fruits and vegetables was associated with a decreased risk of dementia and AD compared with no or low intake (Hughes et al., 2010). High intake of beta-carotene, flavonoids, vitamins C and E, thiamine, and folate from dietary fruit and vegetables was also associated with a lower risk of AD in the Rotterdam Study (Engelhart et al., 2002). Data from the 3C Study demonstrated that a diet rich in fish, n-3 PUFA rich oils, fruits, and vegetables could contribute to decrease the risk of dementia and AD in older persons whereas consumption of n-6 rich oils could exert detrimental effects when not counterbalanced by sufficient n-3 intake. These effects seem more pronounced among APOE ε4 noncarriers (Barberger-Gateau et al., 2007). In the prospective Nurses' Health Study with a follow-up from 1976 to 2003, fruit and vegetable intake was related to cognitive function and decline among aging women. Women with the highest quintile of cruciferous vegetables declined slower compared with the lowest quintile (Kang et al., 2005). These findings were consistent with those from a subgroup of subjects participating in the Chicago Health and Aging Project (CHAP), which found that a high vegetable consumption was associated with a slower rate of cognitive decline over 6 years (Morris et al., 2006). Recently, the hypothesis that combinations of certain nutrients could provide clinically relevant benefits

to patients with AD was demonstrated in the randomized, double-blind, controlled multicenter trial patients with mild AD who consumed the medical food Souvenaid for 12 weeks, which is a multinutrient drink (Scheltens et al., 2010). In several cross-sectional studies, moderate consumption of alcohol, from up to 1 drink per day (up to 14 g of alcohol) to 4 drinks per day (52 g of alcohol), has been associated with a better function in many cognitive domains as compared with no consumer (Carmelli et al., 1999; Elias et al., 1999; Bond et al., 2001, 2003). A very recent meta-analysis included 15 prospective studies (follow-ups ranged from 2 to 8 years), with samples including 14,646 participants evaluated for AD, 10,225 participants evaluated for vascular dementia, and 11,875 followed for any type of dementia (Anstey et al., 2009). This meta-analysis indicated that light to moderate alcohol intake was associated with a 25%–28% reduction in risk of AD and any dementia compared with no drinkers. The role of alcohol for the cognition might be modified by the presence of the APOE ε4 allele (Carmelli et al., 1999; Dufouil et al., 2000) Regarding different types of beverages, several studies suggested that low to moderate wine consumption may be protective against dementia and cognitive decline (Panza et al., 2009). Wine consumption may exert a protective effect, either through alcohol intake itself, through the antioxidant effects of polyphenols richly represented in red wine (Panza et al., 2009) or through both. Red-wine polyphenols are a complex mixture of flavonoids (mainly anthocyanins and flavan-3-ols) and nonflavonoids (such as resveratrol and gallic acid). Flavan-3-ols are the most abundant with oligomeric and polymeric procyanidins (condensed tannins) often representing 25–50% of the total phenolic constituents (Waterhouse, 2002). Given the increasing body of evidence suggesting that AD may be influenced by vascular factors (Panza et al., 2006), it may be concluded that the vascular protection associated to wine consumption decreases the risk of dementia/AD. In fact, in a 5-year follow-up Personnes Agées QUID (PAQUID) cohort, a significant inverse association between flavonoid intake and the risk of dementia was found (Commenges et al., 2000). It has been also suggested that the antioxidant properties of the flavonoids in wine may help to prevent the oxidative damage implicated in dementia. Resveratrol (trans-3, 4, 5-trihydroxystilbene) is the most relevant and the main biologically active nonflavonoid compound found in grapes and red wine (Fig. 10.2). A number of studies have demonstrated the antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic effects of this compound (Jang et al., 1997; Anekonda, 2006). At cellular levels, resveratrol could protect PC12 cells against Aβ-induced toxicity and accumulation of intracellular reactive oxygen species (ROS) (Jang and Surh, 2003). The inhibition of Aβ secretion by resveratrol could be implicated in this neuroprotective effect since the secretion of Aβ is reduced in two cell lines, HEK 293 and N2a, transfected with APP695 and treated with resveratrol (Marambaud et al., 2005). This effect was not mediated by β and γ-secretases activities but may be through the elevation of degradation of Aβ peptide. Very recently, it has been demonstrated that among the five conformers of Aβ (monomers, soluble oligomers, nontoxic oligomers, fibrillar intermediates, and amyloid fibrils), resveratrol can recognize and remodel three of these conformers—soluble oligomers, fibrillar intermediates and amyloid fibrils—into an alternative aggregated species that is nontoxic, high-molecular weight, and unstructured (Ladiwala et al., 2010). Surprisingly, resveratrol does not remodel nontoxic oligomers or accelerate Aβ monomer aggregation. Other neuroprotective mechanisms involve modulation of nuclear factor-kappa B (NF-κB)/Sirt1 pathways since *in vitro* and *in vivo* studies have shown that resveratrol is a specific activator of Sirt1 (Kaeberlein et al., 2005; Baur et al., 2006). This property could be implicated in the protective effect against Aβ involving the inhibition of the NF-κB activity (Chen et al., 2005). Besides its antioxidants effects, the efficacy of resveratrol against Aβ

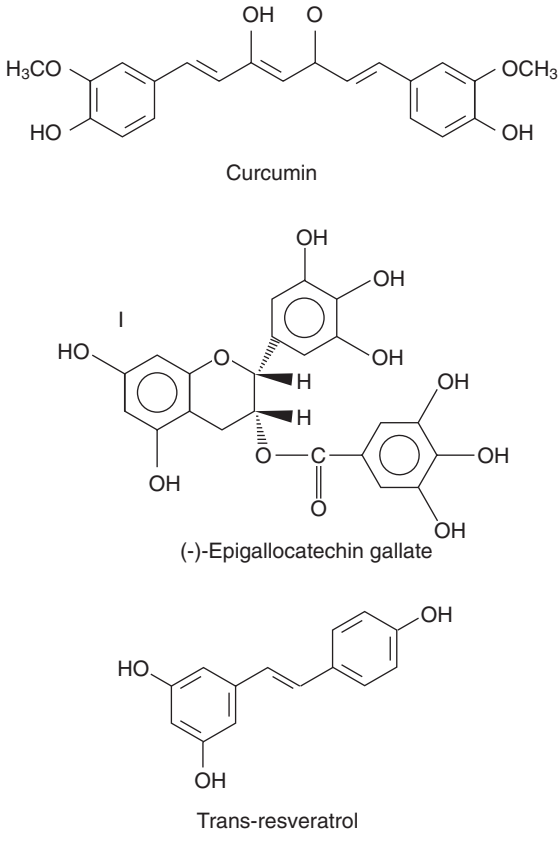

Figure 10.2. Chemical structures of some bioactive components.

toxicity involves several pathways. However, the bioavailability of resveratrol needs to be addressed due to its rapid metabolization in liver and intestinal epithelial cells.

Green tea is rich in flavonoids (30% of dry weight of a leaf) (Graham, 1992) and the main compounds are EGCG, (-)epigallocatechin (EGC), (-)epicatechin (EC), and (-)epicatechin-3-gallate (ECG) (Moyers and Kumar, 2004). These flavonoids display antioxidants properties in this order EGCG > ECG > EGC > EC (Guo et al., 1999) (Fig. 10.2). Green tea polyphenols have shown beneficial effects in animal models of stroke/cerebral ischemia, AD and Parkinson's disease. Neuroprotection in ischemia by EGCG may be mediated through reducing iNOS expression, peroxynitrite formation, preservation of mitochondrial complex activity and integrity, or ferric iron chelation (Suzuki et al., 2004; Mandel et al., 2005; Sutherland et al., 2005). There are several *in vitro* studies that suggest green tea extract could protect neurons from the Aβ-induced damages (Choi et al., 2001; Levites et al., 2003; Bastianetto et al., 2006). Different mechanisms could be involved in this neuroprotective effect since in neuronal cell cultures, EGCG could promote the non-amyloidogenic α-secretase pathway (Levites et al., 2003; Ono et al., 2003). In primary neuronal cells derived from transgenic mice model overexpressing APP with the mutation Sweden, EGCG significantly reduced Aβ peptide generation ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) by 38% (Rezai-Zadeh et al., 2005). EGCG also modulates a number of signaling pathways such as

mitogen-activated protein kinase (MAPK) (Mandel et al., 2008), protein kinase C (Levites et al., 2003), and phosphatidylinositol-3-kinase (PI-3 kinase)-Akt (Koh et al., 2003), and these modulations may mediate some of the neuroprotective mechanisms of EGCG. EGCG, at doses of 1–10 μM, protected against Aβ peptide-induced cell death by activation of protein kinase C (Levites et al., 2002, 2003) that plays a central role in neuronal cell survival, and loss of its activity is frequently observed in neuronal insults such as in the presence of Aβ peptide accumulation and other neurotoxins (Maher, 2001) In summary, green tea and its active component EGCG exert several intracellular mechanisms relating to neuroprotection. However, the translation into clinical use has been problematic primarily as a result of poor oral bioavailability and inefficient delivery to the brain (Feng, 2006) mostly due to poor absorption and intestinal metabolism (Cai et al., 2002).

Curcumin, a polyphenol derived from the rhizome of the herb *Curcuma longa* has a wide variety of biological and pharmacological activities as described above. Chemically, curcumin is a 1,3,6-unsaturated bis 4,6-diketone (Fig. 10.2). The keto groups could exhibit keto-enol tautomerism. Curcumin is a low-molecular-weight compound with potent antioxidant and anti-inflammatory activities. The yellow curry spice is part and parcel of Indian vegetables. When fed to aged Tg2576 mice, curcumin reduced Aβ levels and plaques. Interestingly, curcumin also blocked Aβ aggregation and fibril formation *in vitro* (IC_{50} = 0.8 μM), and this property could be implicated in the reduction of amyloid plaques burden observed *in vivo* after curcumin treatment of Tg2576 mice (Yang et al., 2005). Curcumin is also a good inhibitor of expression of inflammatory cytokines, COX-2 and iNOS likely by inhibition of JNK/AP-1 and NF-κB mediated gene transcription (Aggarwal et al., 2003). All of these factors (IL-1, TNFα, COX-2, iNOS, JNK, NF-κB) are also implicated in Aβ toxicity (Craft et al., 2006; Hoozemans et al., 2006). Moreover, curcumin could chelate the redox active metals iron and copper (Baum and Ng, 2004). However, the effects of these phenolic compounds on Aβ aggregation remain controversial likely due to the limited bioavailability and the metabolism of these compounds (Singh et al., 2008). Clinically, it is considered extremely safe while administered at very high doses. However, it has restrictive pharmaceutical role because of its extremely low aqueous solubility, rapid systemic elimination, inadequate tissue absorption, poor absorption from the gut, and degradation at alkaline pH, which severely curtails its bioavailability (Wahlstrom and Blennow, 1978; Anand et al., 2007).

For instance, only negligible amounts of curcumin in blood plasma were observed after its oral administration with 1 g/kg in Sprague Dawley rats due to its low absorption from the gut (Wahlstrom and Blennow, 1978). Besides this, when curcumin was given orally at a dose of 2 g/kg to rats, a maximum serum concentration of 1.35 (0.23 μg/mL was observed at time 0.83 hour, whereas in humans the same dose of curcumin resulted in either undetectable or extremely low (0.006 ± 0.005 μg/mL at 1 hour) serum levels (Shoba et al., 1998). A very recent study by Yang and collaborators showed that 10 mg/kg of curcumin given i.v. in rats gave a maximum serum curcumin level of 0.36 ± 0.05 μg/mL, whereas a 50-fold higher curcumin dose administered orally gave only 0.06 ± 0.01 μg/mL maximum serum level in rat (Yang et al., 2007). Moreover, the percentage of curcumin absorbed (60–66% of the given dose) remained constant regardless of the dose, indicating that administration of more curcumin does not result in higher absorption (Ravindranath and Chandrasekhara, 1981) In mice, a curcumin dose of 0.1 g/kg via i.p. route showed that only a trace amount (0.4 μg/g) was found in brain tissue (Pan et al., 1999). In humans, an oral absorption of 3.6 g and 4.8 g of curcumin induced peak plasma levels of 11.1 nmol/L and 0.41–1.75 μM, respectively, after 1 hour of dosing (Cheng et al., 2001; Sharma et al., 2004).

Thus, these studies clearly indicate that the serum levels of curcumin in rats and humans are not directly comparable (see reviews by Anand et al., 2007).

10.4 TARGETED NPS FOR DELIVERY OF BIOACTIVES COMPOUNDS FROM FOODS FOR THE TREATMENT OF AD

Fruits and vegetables are considered as rich sources of some essential dietary micronutrients, fibers, and are recently recognized as important sources for a wide variety of phytochemicals, that individually or in combination may have some health benefit effect. Therefore, they have been conferred the status of *functional foods*. Polyphenolic compounds are the most abundant antioxidants and bioactive compounds present in foods, vegetables, and beverages. As described above, some polyphenolic compounds could protect cells against Aβ-induced toxicity or decrease the amyloidogenic pathway. However, most of them are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. Polyphenols present as aglycones can be absorbed from small intestine but most are present in the form of esters or glycosides. Glycosylation influences the chemical, physical, and biological properties of the polyphenols and their absorption by small intestine (Rice-Evans et al., 1996; Scalbert and Williamson, 2000). Moreover, once absorbed, polyphenols are subjected to three main types of conjugation: methylation, sulfation, and glucuronidation. Catechol-*O*-methyl transferase (COMT) catalyzes the transfer of methyl group to polyphenols having a catechol moiety. The concentration of polyphenols is usually very low in plasma after the intake of nutritional dose, except for tea catechins (up to 77% for EGCG) (Lee et al., 2002). Plasma concentration and half-life reached after polyphenol consumption vary highly according to the nature of polyphenols and the food source. For instance, resveratrol has a short initial half-life (8–14 minutes for the primary molecule) (Asensi et al., 2002; Marier et al., 2002) and is metabolized extensively in the body. Wall and collaborators (Walle et al., 2004) showed that the bulk of an intravenous dose of resveratrol is converted to sulfate conjugates within 30 minutes in humans. Thus, bioavailability and pharmacokinetics of polyphenolics is governed by plethora of factors that is its native form (glycosylated/aglycone) and the type of sugar moiety present and its physiochemical properties. Recently, targeted delivery systems by NPs technology have emerged as promising solutions to increase the bioavailability of therapeutic agents. Delivery systems may have considerable benefit by protecting essential nutrients and bioactive food components during food processing and digestion. In this context, NP-loaded polyphenols is an active area of research to increase the oral bioavailability of poorly absorbed phenolic compounds such as curcumin, catechins, or resveratrol.

10.4.1 Catechins coupled with NPs

Promising preclinical data were obtained with the use of green tea polyphenol, (EGCG) in transgenic mice model (Rezai-Zadeh et al., 2005, 2008). While the antioxidant properties of EGCG are well-known, it has been shown to be able to promote nonamyloidogenic processing of APP by upregulating α-secretase, thus preventing brain Aβ plaque formation. Several studies have reported that the green tea polyphenols undergo methylation, glucuronidation, sulfation, and ring fission metabolism (Li et al., 2001; Lambert and Yang, 2003). Moreover, the oral bioavailability of catechins is poor, with only 0.1–1.1% of the

administered dose reported in human studies (Chow et al., 2003). Therefore, nanolipidic EGCG particles using cosolubilization methodology with different ratios (1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32) of nanocarrier material to EGCG on a milligram/milligram basis were prepared (Smith et al., 2010). The formulated NPs were tested in murine neuroblastoma cells that were stably transfected with the human APP gene (APP; SweAPP N2a cells). It was observed that among all the formulations tested, 1 : 8 and 1 : 16 NanoEGCG formulations promoted enhanced levels of α-secretase activity even at the lowest EGCG concentration tested. The prepared formulations were also tested in male Sprague Dawley rats and were delivered via oral gavage at a dosage of 100 mg EGCG/kg body weight. Blood samples were collected at regular time intervals, and the pharmacokinetic parameters were determined. The oral bioavailability of the formulations *in vivo* was observed to be enhanced by more than twofold as compared to the free EGCG in 10% ethanol solution after 5 and 10 minutes. The relative bioavailability has defined by area under the curve, of the NanoEGCG, was 2.31 and 2.5 for the 1 : 16 and 1 : 8 fold, respectively, as compared to the free EGCG. Recently, it has been demonstrated that encapsulation of catechins and EGCG in chitosan-NPs can significantly enhance their *in vitro* intestinal absorption (Dube et al., 2010). Results on the brain uptake of NanoEGCG will be important to evaluate.

10.4.2 NPs targeted with ApoE containing curcumin

As described above, one of the major observations related to curcumin studies involves its extremely low serum levels. Mulik et al. (2010) prepared apolipoprotein E3 mediated poly(butyl) cyanoacrylate NPs containing curcumin (ApoE3-C-PBCA) aimed at enhancing its photostability and cellular uptake. Prepared NPs were characterized for particle size, zeta potential, entrapment efficiency, and *in vitro* drug release. The mean particle size of curcumin loaded PBCA NPs (C-PBCA) and ApoE3-C-PBCA was found to be 178 ± 0.59 nm and 197 ± 2.3 with polydispersity index of 0.24 and 0.18, respectively. A low polydispersity index is a measure of the uniformity of particle size distribution. As observed by TEM the NPs were spherical in shape. The zeta potential of C-PBCA and ApoE3-C-PBCA was observed to be −28.33 ± 0.16 and −22.44 ± 2.3 mV, respectively and thus indicated the stable nature of formed particles. X-ray diffraction analysis was done to confirm the entrapment of curcumin inside the NPs. Results on SH-SY5Y neuroblastoma cells indicated higher efficacy of ApoE3-C-PBCA against Aβ-plaques induced cytotoxicity as compared to plain curcumin solution. Antiapoptotic activity of curcumin was also studied using flow cytometry assays. From the results of all the experiments conducted, it was demonstrated that the properties of curcumin was increased with ApoE3-C-PBCA as compared to plain curcumin solution, thereby suggesting the enhanced cellular uptake and sustained drug release effect. Besides this, the authors also studied the synergistic effect of ApoE3 and curcumin because ApoE3 also possesses both antioxidant and anti-amyloidogenic activity. It was observed that ApoE3 had activity against Aβ-induced cytotoxicity when used with curcumin. Therefore, the attachment of ApoE3 to C-PBCA NPs clearly increased the uptake of curcumin into the SH-SY5Y human neuroblastoma cells, thereby resulting in an enhanced activity compared to plain solution or non-targeted NPs.

ApoE4 is a well-known risk factor for AD while ApoE2 and E3 have protective action. The isoforms of apolipoprotein E (Apo E2, E3, and E4) form complexes with Aβ and display different transport behaviors. Complexation with ApoE2 and ApoE3 prevents transport of Aβ across the blood–brain barrier (BBB) whereas apoE4 promotes the uptake

and accumulation of Aβ in the brain. Transport of ApoE across BBB was studied previously (Martel et al., 1997). All three apolipoprotein E isoforms, that is, ApoE2, ApoE3, and ApoE4, have shown low cerebrovascular uptake with undetectable blood-to-brain transport. However, a significant uptake of ApoE3 and ApoE4 was observed by the choroid plexus. This suggested that ApoE isoforms could enter the cerebrospinal fluid (CSF) by crossing the choroidal epithelium and ultimately be taken up by brain parenchymal cells by low-density lipoprotein receptor (LDL-R)-mediated endocytosis (Martel et al., 1997). Future *in vivo* studies using animal models of AD will highlight the effectiveness of proposed targeted drug delivery system. The uptake into the brain endothelial cells of the NPs covalently bound ApoE as a targetor was also investigated by Zensi et al. (2009). For this, human serum albumin (HAS) NPs was prepared by desolvation technique with and without covalently bound ApoE. The particle size of the resulting ApoE-modified particles was found to be 249 nm. The concentration of the nanoparticulate suspension was estimated to be 20 mg NPs/mL suspension. HSA NPs with polyethyleneglycol (PEG) chains and without ApoE on their surface were prepared by linking mPEGSPA-5000 to the particle surface and were used as controls. The diameter of the control particles was observed to be 208 nm. For *in vivo* studies 10 μL NPs suspension, containing 200 μg of NPs, per gram body weight was injected intravenously into SV 129 mice. After 15 and 30 minutes, mice were sacrificed, and six samples of each brain corresponding to five different brain regions (olfactory bulb, cortex, striatum, hippocampus, cerebellum, and brain stem) were then prepared for electron microscopy analysis. It was found that only those NPs, which had covalently bound ApoE, were detected in brain capillary endothelial cells and neurones, whereas there was no uptake of NPs without ApoE into the brain. Besides this, the ApoE-modified NPs could be detected in all six investigated brain regions. In contrast to the ApoE-NPs, very few pegylated HSA NPs without attached ApoE were observed in the endothelial cells. No particles appeared in the brain tissue after a 30-minute exposure. The authors thus suggested that the particles without ApoE were being taken up unselectively as a rare event and were retained within the endothelial cells, possibly within the lysosomal compartment. Those from *in vitro* studies further confirmed results of *in vivo* studies.

The cellular uptake, cellular binding and cell viability studies were also conducted for HSA/ApoE NPs on mouse brain endothelial cells b.End3. For cellular binding studies, cells were treated with 0.1 mg/mL of the different nanoparticulate formulations for 1 hour and 4 hours, respectively. Flow cytometry analysis was performed for demonstrating the cellular binding of NPs. The cellular uptake of the nanoparticulate formulations was investigated by electron microscopy after incubating the cells for 2 hours with nanosuspensions (1 mg/mL), whereas the cell viability was determined in b.End3 cells using a WST-1 assay after incubation of the cells with ApoE-modified NPs (ApoE-NP) or control NPs without ApoE modification (PEG-NP) at different concentrations (0.1 mg/mL, 1 mg/mL, and 2 mg/mL) for 24 hours. Flow cytometry analysis clearly demonstrated a specific cellular binding of the ApoE-modified NPs as compared to only a marginal binding of the control NPs. Based on the results of the cell viability studies, cytotoxic effect of these nanoparticulate formulations was excluded. The transmission electron micrographs demonstrated the appearance of many dark spheres with characteristic NPs appearance in cells after 2 hours of incubation with a 1 mg/mL NPs suspension. The HSA-NPs appeared to be within intracellular structures surrounded by a membrane, possibly lysosomes. It was concluded from the findings that covalently bound ApoE NPs were taken up into the cerebral endothelium by an endocytic mechanism followed by transcytosis into brain parenchyma.

10.4.3 Resveratrol-loaded NPs protect againt Aβ-induced toxicity

A number of studies have demonstrated the antioxidant and anti-inflammatory of resveratrol. However, clinical studies demonstrated only its minimal effect in humans and this was related to its pharmacokinetics (Mancuso et al., 2007) because in plasma its concentration is very sparse (only several micromoles after addition) with short half-life (Athar et al., 2007). Moreover, the antioxidant role of resveratrol seems to be considerably diluted *in vivo* due to its extensive and rapid metabolism to glucuronic and sulfate metabolites of resveratrol (Anekonda, 2006). Lu et al. (Lu et al., 2009) have investigated the resveratrol loaded-NPs obtained from polycaprolactone (PCL) as the hydrophobic core and PEG as the hydrophilic shell for their good drug encapsulation ability. After 48 hours, NPs containing equivalent doses of 10 μM of resveratrol could protect PC12 cells against Aβ while the free resveratrol did not. Moreover, in contrast to free resveratrol, resveratrol-loaded NPs were nontoxic to PC12 cells after 48 hours of treatment. However, feasibility and advantages of *in vivo* applications of resveratrol-loaded NPs should be investigated.

10.5 CONCLUSION

There is growing evidence that oxidative damage is implicated in the pathogenesis of AD. Several cross-sectional studies have indicated a relationship between blood concentrations of antioxidant micronutrients, polyphenols consumption, and cognitive impairment. However, most of them are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. Over the last decade, there is a general good appreciation of the potential benefits of nanotechnology for the prevention and treatment of some chronic diseases such as AD. NPs combined with nutraceutical compounds or polyphenols could be considered as the promising strategy to deliver bioactive compounds beyond the BBB for the prevention or treatment of neurodegenerative disorders such as AD. Although experimental data have demonstrated effective transport of drugs across BBB using NPs, still there is a need to optimize this general strategy, in terms of efficiency, specificity, and safety. Due to their particular physicochemical properties, such as large surface area, the NPs may cause neurotoxicity after reaching the brain, thus arising a need for further investigations regarding the toxic pathways through which NPs may exert their toxic effects *so* that they can be safely and effectively used. Certain NPs are not easily eliminated by clearance systems *and* could accumulate within the brain to elicit further cytotoxicity *as* published in some recent reports *which* have shown that the NPs could cause injury once they get into the brain (see review by (Medina et al., 2007; Sharma, 2007; Hu and Gao, 2010).

The effects of polyphenols on memory and cognition has recently been summarized by Spencer (2010).

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11 Cancer Prevention by Polyphenols: Influence on Signal Transduction and Gene Expression

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11.1 INTRODUCTION

Cancer is a life-threatening disease and a public health concern worldwide. Breast cancer is the most frequently diagnosed cancer among women. Globally, breast cancer accounts for about 22% of all new cancer cases diagnosed each year among women (www.cancer.ca). Changes in lifestyle and diet are known to affect cancer risk (Irwin et al., 2005). Despite an intense program on cancer research and cancer management over the last few decades, therapeutic options for many cancers are still not efficient, with low specificity of the treatment procedures and causing severe side effects in the patients. A major challenge is to develop new preventive and therapeutic procedures for most cancers. This requires a thorough study of the molecular pathways involved in various malignancies and more importantly, the translation of research results from basic cancer research into dietary preventive strategies. Thus, it is of great importance to develop cancer-preventive strategies and enhance the quality of life.

11.2 GENETIC MECHANISMS OF CARCINOGENESIS

Carcinogenesis is a multistep process, and has been shown to consist of two distinct stages, the preneoplastic and the malignant stages (Moolgavkar and Knudson, 1981). It begins with initiation, when carcinogen-induced genetic damage occurs in cells which then gain growth advantage over normal cells due to the activation of proto-oncogenes and/or loss or inactivation of tumor suppressor genes. This step is irreversible and involves conversion of a normal cell to a premalignant cell. The proto-oncogenes encode proteins that act in the signal transduction pathways and are involved in the control of normal growth and differentiation (Cantley et al., 1991). Therefore, if dominant mutations occur in these genes, or if the genes are overexpressed, abnormalities in growth, control, and differentiation

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occur. In general, oncogenes code for growth factors, receptor tyrosine kinases, or factors involved in mitogenic activity (Bishop, 1991). Oncogenes are reported to be amplified in a significant number of breast cancers (Van de Vijver, 1993). These include members of the *neu* or c-*erb*B-2 gene, c-*myc* gene, epidermal growth factor (EGF)-receptor gene, insulin-like growth factor-1 (IGF-1) receptor gene, and so on. The *neu* gene is known to encode a transmembrane 185-kDa phosphoglycoprotein with tyrosine kinase activity and is a member of the human epidermal growth factor receptor (EGFR) gene family (Akiyama et al., 1986). The c-*erb*B-2 gene is overexpressed in 15–40% of breast carcinomas (Aziz et al., 2001). Amplification of the c-*myc* gene is associated with a high rate of early relapse in node-negative breast cancers (Dosseto et al., 1992). Amplification of the EGF-receptor gene and overexpression of EGF-mRNA and protein has also been observed in several metaplastic breast carcinomas (Filho et al., 2005).

There are five major classes of proto-oncogene/oncogenes: (1) growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin; (2) growth factor receptors: c-*erb*B-2; (3) signal transducers: c-*ras*; (4) transcription factors: c-*myc*, c-*fos*; and (5) programmed cell death regulators: *bcl-2*. Transformation generally occurs as a result of cooperative action of these oncogenes (Hunter, 1991).

Transformation also occurs due to recessive mutations in certain genes, which are called anti-oncogenes or tumor suppressor genes. These are normal cellular genes that encode proteins involved in inducing cell death or apoptosis. Some examples of loss of tumor suppressor gene function leading to the development of breast cancer include that of the p53 gene (Varley et al., 1991), retinoblastoma gene (Fung and T'Ang, 1992), prohibitin gene (on chromosome 17q), and the DCC (deleted in colorectal carcinoma gene, CRC1, CRCR1) located on (chromosome 18q) (Van de Vijver, 1993). The p53 gene encodes a 53-kD phosphoprotein that binds to DNA in a dimer form. Point mutations on the conserved regions of this gene cause the loss of its function and the formation of a protein with altered growth regulatory properties (Varley et al., 1991). Inactivation of the retinoblastoma gene, which encodes a 105-kD phosphoprotein that binds to DNA and regulates cell growth has also been found in 20% of breast carcinomas (Fung and T'Ang, 1992).

The initiated cells have a decreased responsiveness to intercellular and intracellular signals that regulate cell growth, such as negative growth factors, inducers of terminal cell differentiation, and/or programmed cell death (Harris, 1991). Initiation is followed by tumor promotion which involves epigenetic mechanisms such as DNA methylation and histone modification (Jones and Baylin, 2002) and results in increased proliferation and survival of initiated cells leading to the appearance of benign tumors. The second irreversible step in the carcinogenic process is termed conversion. The conversion step leads to the formation of the first malignant cell, and this is followed by a non-rate-limiting process of visible tumor formation, called as progression. Tumor progression leads to further evolution of this tumor to greater degrees of malignancy. Progression is associated with the progressive acquisition of mutations in oncogenes and tumor suppressor genes and chromosomal abnormalities such as monosomy and trisomy (Solomon et al., 1991).

Breast cancer is the most common neoplastic disease afflicting females in Western countries. The major types of breast cancers observed include ductal carcinomas, lobular carcinomas, inflammatory carcinomas, and Paget's disease (Henderson et al., 1997). Among all the major types of breast cancers, the carcinomas or malignant tumors of the epithelium are the most frequent, having incidences ranging from 47% to 75% (Van de Vijver, 1993). There are several risk factors associated with increased incidence of breast cancer. They may be endogenous, and dependent on the genetic constitution of the individual (or hor-

mones), or exogenous, and dependent on the environment in which the individual lives, as well as lifestyle factors. Endogenous factors include age over 50, a family history of breast cancer, early menarche and late menopause, first childbirth after age 30, or nulliparity (Scanlon, 1991; Althuis et al., 2003; Boyd et al., 2003). Women who previously had cancer of the colon, thyroid, endometrium, or ovary also have a higher risk of developing breast cancer (Dobernack and Garcia, 1988). Exogenous factors include high doses of radiation to the chest before age 35, obesity, and dietary factors such as a high-fat diet (Howe et al., 1990; Garofalo et al., 2006). However, a majority of women who develop breast cancer do not have known risk factors, which indicates that the causes may be multiple and quite probably, many risk factors have yet to be identified.

Primarily, treatment of breast cancer includes surgery, radiation, chemotherapy, and hormonal modification (Guarneri and Conte, 2004). However, the existing treatments have drawbacks in that the normal cells are affected in great numbers along with the malignant cells, leading to severe complications including side effects. What becomes clear from previous studies is that one must be able to point out the differences between normal and transformed cells at molecular and biochemical levels to define strategies for treatment and successfully target cancer cells.

11.3 BIOCHEMICAL MECHANISMS OF CARCINOGENESIS

11.3.1 Pathways and signals involved in neoplastic cell transformation and carcinogenesis

The biochemical mechanisms of cell transformation are better understood due to the recent progress in the study of the biochemical relations between *oncoproteins*, the protein products of oncogenes. The oncoproteins send signals from the cell surface to the nucleus by various pathways that are generally termed as signal transduction pathways (Dunn et al., 2005). Any signaling pathway involves signaling cascades, intracellular messenger molecules, and transcription factors. Normal signal transduction is a process by which information from a stimulus outside the cell is transmitted from the cell membrane (e.g., through its receptor) into the cell and along an intracellular chain of signaling molecules to stimulate a response. In cancer cells, the receptors are permanently activated and the signal transduction process occurs continuously even in the absence of a growth regulator or external signal. Defective signal transduction is often associated with aberrant cell cycle regulation, which in turn can lead to the development and progression of cancer (Vivanco and Sawyers, 2002). Several signal transduction pathways such as the extracellular signal-regulated kinase/mitogen-activated protein kinase (Erk/MAP) pathway and the phosphatidyl inositol 3-kinase (PI 3-kinase) pathway have been implicated in mammary carcinogenesis (Vivanco and Sawyers, 2002).

The MAP kinase pathways transmit and integrate signals from diverse extracellular stimuli, and translate them into different cellular responses that may include proliferation, differentiation, or apoptosis (Bode and Dong, 2005). MAP kinases are activated by translocation to the nucleus, where they phosphorylate a variety of target transcription factors important in tumor development, including AP-1 and NF-κB (Sanchez et al., 1994). These in turn may activate transcription of a variety of cancer-related genes such as cyclooxygenase-2 (COX-2), c-Myc, BRCA1. The three groups of MAP kinases are extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases

(JNKs/SAPs), and p38 kinases, and are activated by dual phosphorylation on threonine and tyrosine at T-X-Y motifs. MAPK is phosphorylated by MAP kinase kinases (MAPKKs), and they are in turn activated by MAPKK kinases (MAPKKKs). ERKs play a role in transmitting signals initiated by tumor promoters such as EGF, and PDGF (Cowley et al., 1994), whereas JNKs/SAPKs and p38 kinases are induced by stress.

The phosphatidylinositol-3 (PI-3) kinase signaling pathway is another pathway that has been implicated in malignant transformation including breast cancer in several studies (Krasilnikov, 2000; Fry, 2001). Activation of PI-3 kinase has been shown to lead to the production of several potential lipid signaling second messenger molecules (Stephens et al., 1993). Downstream effectors of PI-3 kinase pathway include protein kinase C (PKC), Akt (protein kinase B), ribosomal p70S6 protein kinase (p70S6K), and the mammalian target of rapamycin (Mtor). Akt is overexpressed in many cancer types and is associated with increased tumorigenicity (Cheng et al., 1992; Bellacosa et al., 1995). p70S6K is shown to be overexpressed in breast cancer cells (Bärlund et al., 2000). Activation of p70S6K requires Mtor (Brown et al., 1995), and Mtor is directly phosphorylated by Akt. Nomura et al. (2003) showed that blocking Mtor or Akt activity suppressed EGF-induced cell transformation in JB6 cells.

Transcription factors are downstream effectors of these signal transduction pathways, and target a diversity of genes related to various cellular events. Deregulation of transcription factors and signal transduction pathways causes altered gene expression that leads to imbalanced proliferation, differentiation, apoptosis, and ultimately malignant transformation. The role of transcription factors such as AP-1, Myc, ER (estrogen receptor), and STAT (signal transducers and activators of transcription) in mediating breast tumorigenesis has been summarized by Benz (1998).

Signals may be extracellular, intracellular, or intercellular, and include (1) endocrine: hormone synthesized in endocrine cells and exported via exocytosis into the extracellular medium, for example, blood, (2) paracrine: hormone reaches the target cells by passive diffusion, and (3) autocrine: hormone produced by the signaling cell affects a cell of the same type.

11.3.2 Extracellular signal transduction

The "extracellular" signaling molecules include hormones and growth factors that bind to receptors and activate their ligand-binding sites on the outside of the membrane. This causes a change in the shape or conformation of the receptor on its intracellular domain and leads to triggering of events inside. Receptors typically respond only to the specific molecule or "ligand" for which they possess high affinity, whereas molecules that are even slightly different tend to have no effect or act as inhibitors (Krauss, 2001c). However, a cell can have several different receptors that recognize the same hormone, but activate different signal transduction pathways, or different hormones and their receptors can stimulate the same signal transduction pathway. Also, different tissue types can respond differently to the same hormonal stimulus. The receptors belong to two classes, the "membrane-associated receptors" and intracellular or "cytoplasmic" receptors (Krauss, 2001c). Because of the plasticity of the stimulus-signal transduction systems, it is a complex issue when attempting to control overactive stimulus-signal transduction processes.

Membrane-associated receptors are proteins that span the plasma membrane bilayer with one end of the receptor outside (*extracellular domain*) and one inside (*intracellular domain*) the cell. Upon ligand binding on the extracellular domain, the receptor undergoes a con-

formational change that affects the intracellular domain, leading to further activation of effector proteins. Other membrane-associated proteins are activated in turn, or come together to form a multiprotein complex that finally sends a signal via a soluble molecule into the cell (Krauss, 2001c).

Intracellular (cytoplasmic or nuclear) receptors are soluble proteins localized within the cytosol or nucleus. The ligand passes through the plasma membrane, usually by passive diffusion, to reach the receptor and initiate the signal cascade. The nuclear receptors are often ligand-activated transcription activators (Krauss, 2001c).

Steroid receptors are a subclass of nuclear receptors and are located primarily in the cytosol. In the absence of steroid hormone, the receptors are bound to chaperone proteins (also called as *heatshock proteins* or *Hsp*s) in a complex called *aporeceptor complex* (Krauss, 2001a). The binding of the hormone to this complex leads to activation of the receptor and initiates the translocation of the receptor into the nucleus. The activated receptor binds to the DNA at receptor-specific hormone responsive elements (HREs) that are DNA sequences located in the promoter region of target genes (Krauss, 2001a), and causes transactivation. Steroid receptors can also have a *repressive* effect on gene expression, when their transactivation domain is masked, so they cannot activate transcription (Beato et al., 1995). Furthermore, there is another phenomenon called as *cross talk*, where steroid receptor activity is enhanced by phosphorylation of serine residues at its N-terminal end, as a result of another signal transduction pathway, for example, mitogens phosphorylate the ER through MAP-kinases and modulate the signaling pathway of the steroid hormone receptors (Kato et al., 1995).

11.3.3 Intracellular signal transduction

Intracellular signal transduction is largely carried out by the second messenger molecules. Intracellular signaling molecules in eukaryotic cells include heterotrimeric G proteins, small GTPases, cyclic nucleotides such as cyclic AMP (cAMP) and cyclic GMP (cGMP), calcium ion, phophoinositide derivatives such as inositol-triphosphate (IP_3), diacylglycerol (DAG), and various protein kinases and phosphatases (Krauss, 2001b).

11.3.3.1 Ca^{2+} as a second messenger

Ca^{2+} acts as a signaling molecule within the cell. The concentration of free Ca^{2+} within the cell is usually very low (below micromolar levels); it is stored within organelles, usually the endoplasmic reticulum (sarcoplasmic reticulum in muscle cells) where it is bound to molecules like calreticulin. To become active, Ca^{2+} must be released from the endoplasmic reticulum into the cytosol (Berridge, 1993). Two receptor/ion channel proteins are involved in transport of Ca^{2+} from the internal organelles. The $InsP_3$-receptor is a transmembrane protein, localized in the endoplasmic reticulum, and has two transmembrane domains in the vicinity of the C terminus. The active receptor is composed of four identical subunits. In epithelial cells, membrane-associated signaling pathways such as the PI-3 kinase pathway are activated by ligands involving the activated receptor and phospholipase C (PLC) resulting in the formation of $InsP_3$ and release of Ca^{2+} from storage organelles (Berridge, 1993). The *ryanodine receptor* named after the plant alkaloid ryanodine, is similar to the $InsP_3$ receptor and transports Ca^{2+} into the cytosol by a feedback mechanism; a small amount of

Ca^{2+} in the cytosol near the receptor causes it to release even more Ca^{2+}. This phenomenon is observed in neurons and muscle cells (Berridge, 1993).

Ca^{2+} is required in a variety of processes such as cell proliferation, secretion, cytoskeletal organization, cell motility, gene expression, and metabolism (Schreiber, 2005). The three main pathways that lead to Ca^{2+} activation are the G protein regulated pathways, pathways regulated by receptor-tyrosine kinases, and ligand- or current-regulated ion channels (Krauss, 2001b). Ca^{2+} in turn regulates the activity of proteins either directly or through Ca^{2+}-binding proteins. Calmodulin is a high-affinity intracellular Ca^{2+} receptor and serves as the primary mediator of Ca^{2+}-dependent signaling in eukaryotic cells (Means and Dedman, 1980). It is a ubiquitous, highly conserved protein that plays a critical role in numerous essential cellular functions including Ca^{2+} transport, cell motility, cytoskeletal assembly, protein phosphorylation/dephosphorylation, cell proliferation, and cell cycle progression (Lu and Means, 1993; Takuwa et al., 1995; Chin and Means, 2000). Calmodulin functions independently as well as by Ca^{2+}-dependent activation of specific target enzymes. Abnormalities in Ca^{2+}-calmodulin signaling is known to lead to carcinogenesis (Veigl et al., 1982, 1984; Sheng et al., 1991). This is supported by studies demonstrating a higher level of calmodulin in cancer cells (Wei and Hickie, 1981; Takemoto and Jilka, 1983) as compared to the normal cells. Calmodulin antagonists have been reported to inhibit the proliferation of breast cancer cell lines (Strobl et al., 1994; Jacobs et al., 2000). Tamoxifen, an antiestrogen compound widely used for breast cancer chemotherapy, is also a powerful inhibitor of calmodulin-promoted phosphodiesterase activity (Jordan and Murphy, 1990). Therefore, Ca^{2+}-calmodulin pathway and calcium-mediated processes may be effective targets for cancer prevention.

11.3.3.2 Signal amplification and termination

Signaling pathways commonly amplify the initial signal received by the receptor during the course of the signal transduction. The binding of one or just a few hormone molecules to the receptor can induce a cascade of events resulting in the amplification of the signal. Amplification can occur at several points in the signaling pathway. The extent of amplification, or amplification factor, varies greatly at the different levels of the signal transmission (Krauss, 2001c). An initial amplification often occurs at the level of the hormone-receptor complex (Krauss, 2001c). An activated receptor is capable of activating many downstream effector proteins. The signal amplification at the level of the hormone-receptor complex may depend on factors such as life span of the hormone-receptor complex, or frequency of the reaction with the effector protein (Krauss, 2001c). Termination of signaling pathways involves mechanisms such as protein dephosphorylation which deactivate the hormone-receptor complex, or internalization of the hormone-receptor complex, where a section of the cell membrane, together with the bound proteins, is pinched off and transported to the interior of the cell. The receptor is either returned to the cell membrane or degraded (Wei, 2006).

11.3.3.3 Signal transduction, cell cycle progression, and oncogenesis

When a cell receives a signal from growth factors, cytokines, or mitogens, it is stimulated to divide. The life cycle of a cell is divided into the following phases: G0 phase, where the cell has just emerged from mitosis and is growing to reach its mature stage; G1 phase, the most prolonged stage where the cell does not divide and functions as part of the tissue where it belongs; S phase, where the cell enters the mitotic stage and duplicates its DNA;

and finally the M phase, where the cell enters mitosis. Oncogenic events involving mutation of genes that regulate the cell cycle, lead to deregulation of the cell cycle machinery and result in a shorter proliferation time. For example, the Ras protein is a signal transduction protein that, under proper stimulation (hydrolysis of guanosine triphosphate to guanosine diphosphate), induces the cell to continue its normal cycle. Oncogenic activation of K-ras elicits mitogenic responses and continuous cell stimulation in the absence of a proper stimulus (Agbunag and Bar-Sagi, 2004). One of the critical targets of activated *ras* is MAP kinase. When *ras* becomes activated (in normal or cancer cells), MAP kinase is activated resulting in the expression of cyclin D1 (Bottazzi and Assoian, 1997). Cyclin D1 is found to be overexpressed in many human cancers, including breast and ovarian cancer (Masciullo et al., 1997).

Deregulation of checkpoint proteins could also lead to uncontrolled proliferation (Sherr, 1996). For example, p53 is a tumor suppressor gene that is involved in DNA repair mechanisms. When DNA damage occurs, it triggers a cascade of events that inhibit progression of the cell cycle and lead to cell apoptosis if damage cannot be reversed. Cells with DNA damage rest at the G1-S checkpoint of the cell cycle until the damage is corrected, but cells that lack p53 or have a mutant form do not stop at G1. Also cells that lack p53 do not undergo apoptosis.

11.3.3.4 Oncogenesis and regulation of apoptosis

Apoptosis is one form of programmed cell death. Cancer cells typically acquire an increased resistance to apoptosis due to defects in signal transduction mechanisms (Shibata et al., 1999). Cytoplasmic or nuclear kinases transduce mitogenic signals that lead to cell proliferation and transformation. Activation of such molecules may also inhibit cells from undergoing programmed cell death or apoptosis. In cancer cells, an antiapoptotic mechanism is often activated to rescue the transformed cell from programmed cell death. The most common mechanism is the activation of Bcl-2 (Basal cell lymphoma-2) family of proteins, Bcl-2, Bcl-xl, and Bcl-w (Teixeira et al., 1995) that are able to inhibit cytochrome c release from the mitochondria and rescue the cell from apoptosis. Inactivation of the proapoptotic molecules Bax (BCL2-associated X protein), Bak, Bid, or Bim also contributes to rescuing the cell from apoptosis (Shibata et al., 1999). Activation of oncogenic kinases such as Akt-1 protects cells from apoptosis by inhibiting the proapoptotic molecule Bad (Bcl2-associated protein promoting cell death) (Khwaja, 1999). Several antiapoptotic signals such as growth factors (PDGF, EGF, etc.) lead to the activation of signaling pathways including the PI-3 kinase or MAPK pathways that can also be activated by oncogenic kinases such as Akt and Tpl-2 (Tpl-2 is a MAPKKK involved in ERK/MAPK pathway and is activated during inflammation in response to tumor necrosis factor-alpha (TNF-α) and interleukins; inhibition of Tpl2 is a strategy to prevent inflammation). Thus, activation of these oncogenic kinases rescues the cell from the apoptotic signals and promotes their survival. Thus, signal transduction pathways and signaling molecules play a crucial role in carcinogenesis and are beginning to be recognized as ideal targets in cancer prevention and therapy.

11.4 SIGNALING PATHWAYS IN BREAST CANCER

The occurrence of human breast cancer is associated with the amplification and/or overexpression of a number of genes. Among these are genes that encode growth factors, growth

factor receptors, and transcription factors that are part of the signaling pathways. Overexpression of ErbB2 (type 1 receptor tyrosine kinases belonging to the Erb family with homology to the v-erbB oncogene; having four members Erb-B1, Erb-B2, Erb-B3, and Erb-B4) has been shown to be associated with a considerably increased Erk/MAP kinase pathway activity in breast cancer cell lines (Janes et al., 1994) suggesting that MAPK pathway is involved in mammary tumorigenesis. Other signaling molecules show elevated activity in breast carcinomas relative to normal breast tissues. The nonreceptor tyrosine kinase c-Src and PKC are examples of other signaling molecules implicated in breast cancer (Ottenhoff-Kalff et al., 1992). Sivaraman et al. (1997) have also shown that Erk/MAP kinase activity is high in cancerous breast tissue compared to normal breast tissue.

Intracellular second messenger molecules such as calcium and calmodulin also play a significant role in cell proliferation and carcinogenesis. Calcium homeostasis is known to be dysregulated in cancer cells and elevated levels of calcium and calmodulin are found in cancer tissue as compared to the normal tissue (Wei and Hickie, 1981; Veigl et al., 1982; Takemoto and Jilka, 1983). The role of calcium and calmodulin in proliferation and apoptosis and their potential for serving as biomarkers of cell death are important avenues through which disease prevention may be achieved. As discussed later, the potential for flavonoids to mediate cell death via calcium second messenger signaling is also important in this regard.

11.4.1 Calcium homeostasis and signaling

In a resting cell, the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is maintained at low levels of approximately 100 nM. This is achieved by the action of Ca^{2+} transporters that extrude Ca^{2+} across the plasma membrane to the extracellular environment, or enhance uptake of Ca^{2+} into intracellular organelles such as endoplasmic reticulum (Carafoli, 1987). In contrast, the free extracellular Ca^{2+} concentration is up to 10,000-fold higher at around 1–2 mM (Carafoli, 1987). Upon mitogenic stimulation (growth factors, hormones, etc.), a Ca^{2+} signal in the form of a Ca^{2+} transient is induced and $[Ca^{2+}]_i$ is elevated beyond its resting concentration to micromolar levels. This occurs via two mechanisms; entry of extracellular Ca^{2+} via voltage-or receptor-operated Ca^{2+} channels in the plasma membrane, or the release of stored Ca^{2+} from intracellular organelles via Ca^{2+} channels in internal membranes (inositol-1,4,5-trisphosphate receptors and ryanodine receptors) (Berridge et al., 2000, 2003; Carafoli et al., 2001). Intracellular stores of calcium include the endoplasmic reticulum and the mitochondria where Ca^{2+} is highly concentrated (Nilius et al., 1993; Lepple-Wienhues et al., 1996).

The elevated $[Ca^{2+}]_i$ generates an intracellular Ca^{2+} signal that is modified and decoded by Ca^{2+}-binding proteins to regulate cellular processes (Berridge et al., 2000, 2003; Carafoli et al., 2001). The most studied Ca^{2+}-modulated protein is calmodulin, which is a member of the EF-hand family of proteins. In response to an increase in $[Ca^{2+}]_i$, calmodulin's four EF-hands bind Ca^{2+}, and this induces a conformational change that allows the Ca^{2+}-modulated protein to bind and activate various downstream target proteins. CaM targets include cyclic nucleotide metabolism, signal transduction pathways involving phosphorylation and dephosphorylation, and calcium transport. Once Ca^{2+} has served its signaling function, $[Ca^{2+}]_i$ is lowered to resting levels in normal cells to maintain intracellular Ca^{2+} homeostasis. Ca^{2+} is either sequestered into intracellular organelles by pumps such as the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) or is extruded to the extracellular environment by transporters such as the Na^+/Ca^{2+} exchanger (NCX) or the plasma

membrane Ca^{2+}-ATPase (PMCA) (Berridge et al., 2000, 2003; Carafoli et al., 2001). Mitochondria are important organelles for the sequestration of intracellular Ca^{2+} via the mitochondrial Ca^{2+} uniporter that is located in the inner mitochondrial membrane and is driven by the negative electrical potential across this membrane (Kirichok et al., 2004). When there is intense Ca^{2+} signaling, mitochondria rapidly sequester Ca^{2+}, thereby temporarily helping to prevent excessive increases in $[Ca^{2+}]_i$ that may result in cell death (Carafoli et al., 2001). When signaling has ceased, Ca^{2+} is released back slowly from mitochondria to the cytosol via the mitochondrial NCX, for uptake by the endoplasmic reticulum or removal from the cell (Carafoli et al., 2001).

11.4.2 Role of calcium in regulating cell proliferation and cell cycle

Calcium plays an important role in regulating cell cycle and cell proliferation (Berridge et al., 2000; Munaron et al., 2004). The cell cycle is regulated by the calcium signaling pathway at the G0/G1 transition, where calcium plays a role in regulating the transcription of immediate early genes (Berridge, 1995), and also at late G1 and G2/M, where calcium helps complete the cell cycle by promoting events that lead to cell division. Brears et al. (1999) have shown that Ca^{2+} signaling may be linked with breast epithelial cell proliferation; for example, the activation of MAPK in MCF-7 breast cancer cells by 17β-estradiol involves a Ca^{2+}-mediated pathway involving the release of Ca^{2+} from intracellular stores.

11.4.3 Regulation of the cell cycle by calmodulin

Calmodulin is the primary mediator of Ca^{+}-dependent signaling in eukaryotic cells, and operates as a high affinity intracellular Ca^{2+} receptor. It is a ubiquitous, highly conserved protein that plays a critical role in numerous essential cellular functions including cell proliferation and cell cycle progression (Lu and Means, 1993). Chafouleas et al. (1982) first showed that calmodulin levels doubled at the G1/S boundary in CHO cells. Rasmussen and Means (1989) later demonstrated that calmodulin is essential for cell cycle progression in eukaryotic cells. They prepared clonal lines of mouse C127 cells that contained a chicken calmodulin minigene, so that intracellular calmodulin levels could be manipulated. Increase in calmodulin accelerated cells past the G1/S boundary and G2/M boundary, while a decrease in calmodulin caused cells to become arrested in G1, G2, and metaphase of mitosis (Rasmussen and Means, 1989).

11.4.4 Calcium signaling and cell death

Ca^{2+} has been described as a versatile and ambivalent intracellular messenger (Clapham, 1995; Carafoli et al., 2001). It is a key regulator of cell survival, but at the same time, calcium can also induce apoptosis in response to a variety of pathological conditions. Depending on the subcellular, spatial, and temporal distribution pattern of $[Ca^{2+}]i$ (Berridge et al., 1998), activation of a particular cell survival- or cell death-promoting pathway occurs. Several studies providing a link between intracellular calcium homeostasis and apoptosis have been published (Zhu et al., 2000; Distelhorst, 2002; Ermak and Davies, 2002; Dross et al., 2003). The typical feature of an apoptotic Ca^{2+} signal appears to be a sustained, overall increase in $[Ca^{2+}]_i$ (200–500 nM) that exceeds the cytosolic Ca^{2+} buffering capacity and reaches elevated, cytotoxic levels (Sergeev et al., 2000; Sergeev, 2004).

Apoptosis is one form of programmed cell death. It is characterized by typical morphological and biochemical characteristics such as chromatin margination and condensation, early nuclear collapse, and nucleosomal ladder formation (Zakeri et al., 1995). Although cellular Ca^{2+} has been strongly implicated in induction of apoptosis and regulation of the apoptotic signaling pathways, mechanisms of Ca^{2+} signaling in apoptosis are not clear.

Apoptotic pathways involve activation of cellular caspases (Saraste and Pulkki, 2000), whereas the nonapoptotic pathways do not involve caspases and include mechanisms such as necrosis and autophagy. Necrosis is the result of severe cellular damage, and the morphological features include membrane distortion, organelle degradation, and cellular swelling. Autophagy is a form of nonapoptotic, non-necrotic cell death. It is a process by which the unwanted proteins and organelle components are degraded within lysosomes (Gozuacik and Kimchi, 2004). Though it is a survival mechanism and activated in response to stress, cells that undergo excessive autophagy are induced to die, and the morphology includes membrane blebbing and increased lysosomal activity (autophagic vesicle formation). Defects in these pathways are also associated with tumorigenesis though the molecular mechanisms regulating them are not fully understood (Gozuacik and Kimchi, 2004).

11.4.5 Mitochondria, calcium signaling, and apoptosis

Mitochondria play an important role in cellular calcium signaling and apoptosis (Green and Reed, 1998). The driving force for mitochondrial Ca^{2+} uptake is the mitochondrial membrane potential $\Delta\Psi_m$. The inner mitochondrial membrane is not freely permeable to ions. The uniporter transports Ca^{2+} in the absence of an immediate exchange of ions and helps to buffer huge quantities of Ca^{2+} (Gunter and Pfeiffer, 1990). Due to the relatively low affinity of the uniporter, the global $[Ca^{2+}]c$ rise (~1 μM) established by ER calcium release and calcium entry stimulated by physiological $[Ca^{2+}]c$ signals results in a slow increase in mitochondrial $[Ca^{2+}]m$ levels. However, excessive Ca^{2+} accumulation by mitochondria due to a long lasting and increased $[Ca^{2+}]c$ signal may cause mitochondrial dysfunction (Hoek et al., 1995; Heerdt et al., 1997).

Factors such as oxidative stress, ATP depletion, or activation of the calcium uniporter cause an increase in mitochondrial calcium resulting in dissipation of the mitochondrial membrane potential that leads to an increase in the mitochondrial permeability transition (MPT) and consequently into necrotic or apoptotic cell death (M'Bemba-Meka et al., 2005). This is caused through the release of cytochrome c that commits the cell to die by either a rapid apoptotic mechanism involving Apaf-1-mediated caspase activation, or a slower necrotic process due to the collapse of electron transport (Green and Reed, 1998). Several plant flavonoids have been shown to cause a 10-fold activation of the mitochondrial calcium uniporter at concentrations that could be reached in human plasma under physiological conditions (Montero et al., 2004) and inhibition of F0F1 ATPase (Jianbiao and Ramirez, 2000).

11.5 CANCER PREVENTION AND THERAPY

11.5.1 Targeted therapies

Cancer cells replicate much more quickly than most cells in the body. This was the rationale used for employing chemotherapy to treat cancer. Chemicals that were toxic to rapidly

dividing cells were the agents that interfered with the process of DNA replication. These chemicals were therefore harmful to normal cells with rapid rates of division, such as that of hair, skin, and the cells lining the digestive tract. For several years, these nonspecific chemotherapeutic agents were the only treatment options available to cancer patients.

With the advent of new molecular biology tools, the study of cancer began to be pursued at the molecular and genetic level, especially in the late 1980s and early 1990s, and a new phase in cancer treatment emerged. Research in this area led to a better understanding of the connection between signal transduction and cell cycle control pathways. Proteins within these pathways were identified as potential targets for therapeutic intervention. In theory, targeted therapies have the potential to inhibit the growth of cancer, without causing many of the side effects associated with chemotherapy. Some of the promising targeted therapies that are currently being used and developed for the treatment of cancer include the use of therapeutic antibodies, antisense nucleotides, and cancer vaccines. For example, Herceptin® (Trastuzumab) is a therapeutic monoclonal antibody (Escalante et al., 2006) targeted to the HER-2 protein, a growth factor receptor found at high levels on the surface of certain cancer cells. Approximately 25–30% of women with breast cancer are HER-2 positive, and have a more aggressive form of the disease. Herceptin binds to a specific region on HER-2 and promotes internalization of HER-2 resulting in migration of the receptor to the inside of the cell, so HER-2 can no longer stimulate cancer cell growth by amplifying the effect of other growth factors. Herceptin is approved for first-line use in combination with paclitaxel, and as a second- and third-line treatment as a single agent for patients with HER-2 positive metastatic breast cancer (Brenner and Adams, 1999).

Another example is the cancer vaccine developed against cervical cancer by Glaxo Smith-Kline and Merck. The HPV (Human Papilloma Virus) bivalent vaccine contains viral proteins from two HPV types: HPV 16 & 18, the types that account for about 70% of the worldwide cases of cervical cancer. However, designing such drugs is difficult and time-consuming, and so developing a safe yet effective drug remains a big challenge in cancer therapeutics. Recently, nutritional or dietary factors have attracted a great deal of interest because of their perceived ability to act as highly effective chemopreventive agents. A large body of epidemiological evidence, together with data from animal and *in vitro* studies, strongly supports the cancer-preventive properties of several dietary constituents and phytochemicals (Block et al., 1992). In general, vegetables and fruits, dietary fiber, and certain micronutrients appear to be protective against cancer (Steinmetz and Potter, 1991; Block et al., 1992), whereas fat, excessive calories, and alcohol seem to increase the risk of developing cancer (Hursting et al., 1990; Longnecker, 1994; National Academy of Sciences, 1989). Continued research has provided a clearer understanding of the mechanisms of chemopreventive action of phytochemicals at the molecular and biochemical level. Consumption of functional foods is the most efficient way of obtaining protection against chronic diseases such as cancer, since epidemiological studies have suggested an association between consuming such foods with a decreased cancer risk (Liu, 2004). The potential antitumor efficacy of pure and isolated polyphenols has been demonstrated by several researchers in *in vitro* and *in vivo* systems (Onozawa et al., 1998; Banerjee et al., 2002; Chen et al., 2003). In previous studies (Hakimuddin et al., 2004, 2006, 2008), specific polyphenol fractions from red wine showed selective cytotoxicity toward MCF-7 breast cancer cells, as compared to the nontumorigenic MCF-10A cells and normal human mammary epithelial cells (HMEC). By contrast, authentic flavonoids such as quercetin and naringenin were more toxic to the normal HMEC than cancer cells. Flavonoids have been shown to be specific inhibitors of calcium and calmodulin-promoted biochemical processes

(Nishino et al., 1984; Paliyath and Poovaiah, 1984; Jacobs et al., 2000). Therefore, flavonoids may be able to downregulate potentially abnormal Ca^{2+} second messenger systems in cancer cells. This may involve disruption of calcium homeostasis, energy metabolism and breakdown of cellular compartmentalization leading to apoptosis/ necrosis of the cell.

11.5.2 Phytochemicals and cancer prevention

Epidemiological studies have indicated a positive correlation between fruit and vegetable consumption and cancer prevention (Block et al., 1992). A variety of phytochemicals are found in fruits and vegetables, including terpenes, polyphenols, carotenoids, and sulfur-containing compounds that have the potential to reduce cancer development. The specific mechanisms of cancer-preventive action of these phytochemicals are being uncovered in several studies (reviewed by Jang et al., 1997; Middleton et al., 2000; Yang CS et al., 2001; Heim et al., 2002). For example, resveratrol, a polyphenol found in grapes and wine, exerts chemopreventive activity in U-937 myeloid, HeLa and H4 epithelial cells by induction of phase II carcinogen detoxification enzymes and inhibition of I kappa B kinase activity, the key regulator of NFkappa-B (transcription factor) activation, and signaling enzymes such as PKC (Holmes-McNary and Baldwin, 2000). Sulforaphane, a compound found in cruciferous vegetables such as broccoli, induces apoptosis in SV-40-transformed mouse embryonic fibroblasts through the activation of Bcl-2 family of proteins Bax and Bak, which in turn are translocated to mitochondria causing the release of apoptogenic molecules such as cytochrome-c into the cytosol (Choi and Singh, 2005). Tea polyphenols have been shown to exert cancer chemoprevention through a blockade of mitogenic signals by modulating EGFR function and MAPK cascades (Lin, 2002).

11.5.2.1 Plant polyphenols

Polyphenols are compounds produced by plants as secondary metabolites. They are essential to the physiology of plants and are involved in diverse functions such as lignification and structure, pigmentation, pollination, allelopathy, pathogen and predator resistance, and growth. Polyphenols consist mainly of flavonoids that have a common C_6–C_3–C_6 structure with two aromatic rings linked through an oxygenated heterocyclic ring, and are synthesized by the highly branched phenylpropanoid pathway. Approximately 8000 flavonoids have been described to date (Harborne, 1994), major classes being flavonols, flavones, flavan-3-ols, flavanones, anthocyanins, and isoflavones.

11.5.2.2 Dietary intake and absorption of polyphenols

Due to the immense diversity in forms, there is wide variation on the quantities of polyphenols that are consumed daily throughout the world. Several agricultural and agronomic factors including species, variety, light, the extent of ripeness, processing, and storage, influence differences in concentrations of polyphenols (Peterson and Dwyer, 1998) within the produce, and consumption of more plant based foods provides an increased intake of polyphenols. Kuhnau (1976) first reported that dietary flavonoid intake in the United States was ≈1 g/day and consisted of the following: 16% flavonols, flavones, and flavanones; 17% anthocyanins; 20% catechins; and 45% "biflavones." Subsequently, other studies provided similar data concerning the intake of various classes of polyphenols in different countries.

Flavonol consumption was estimated at ≈20–25 mg/day in the United States, Denmark, and Holland (Hertog et al., 1993; Sampson et al., 2002). Due to high consumption of berries in Finland, average anthocyanin intake was reported to be 82 mg/day (Heinonen, 2001). Consumption of soy products in Asian countries is reported to be ≈10–35 g/day, with a mean intake of 25–40 mg isoflavones/day (Kimira et al., 1998). In Spain, the total consumption of catechins and proanthocyanidin dimers and trimers has been estimated at 18–31 mg/day, and the main sources are apples, pears, grapes, and red wine (de Pascual-Teresa, 1999). Several recent studies report total mean daily polyphenol intakes around 800 mg/day (Saura-Calixto et al., 2007; Ovaskainen et al., 2008; Saura-Calixto and Goni, 2009; Hervert-Hernandez et al., 2011). A lower level of uptake was reported in the U.S. population (Chun et al., 2007).

After intake, polyphenols are released from the plant food matrix by chewing, and broken down by the digestive juices in the gastrointestinal tract, and ultimately the microorganisms of the colon. Properties such as molecular size and configuration, lipophilicity, and solubility, determine the extent of absorption of the flavonoid after its release from food (Hollman, 2004). Most flavonoids are present in the diet usually as *O*-glucosides, except catechins. It was initially believed that only aglycone structures of flavonoids that were obtained after hydrolysis with the intestinal bacterial enzymes could be absorbed. However, Hollman et al. (1995) in a study with ileostomy subjects showed that approximately 52% of quercetin glucosides were absorbed from onions, and this was confirmed in a human pharmacokinetic study (Hollman et al., 1997). It was proposed that the intestinal Na^+-dependent glucose cotransporter (SGLT1) was involved in the transport of quercetin glucosides (Hollman et al., 1995). In contrast, catechins occur in the diet as aglycones and galloylated forms. Results from pharmacokinetic studies in animals suggest that catechin is absorbed from the small intestine in both the aglycone and the galloylated forms (Lee et al., 1995).

11.5.2.3 Bioavailability and tissue uptake

Bioavailability is defined as the rate and extent of absorption by systemic circulation (discussed earlier in Chapter 5 by Jacob and Paliyath). A great deal of information is available on the the bioavailability and tissue uptake of polyphenols from different food sources (Manach et al., 2004, 2005; Scholz and Williamson, 2007; Holst and Williamson, 2008). Depending on the nature of the polyphenol and the food source, plasma concentrations reached after polyphenol consumption has shown to vary to a great extent. Following consumption of 80–100 mg quercetin equivalents from apples, onions, or meals rich in plant products, plasma levels of 0.3–0.75 µmol/L are reached (Hollman et al., 1997; Graefe et al., 2001). Catechin and epicatechin ingested as green tea reach plasma levels of 0.1–0.7 µmol/L for an intake of 90–150 mg, and 0.09 µmol/L after an intake of 35 mg from red wine (Donovan et al., 1999). In contrast, peak concentrations of anthocyanins reached in plasma are at quite a lower level of 0.1–0.3 nmol/ L for an intake of ≈110–200 mg (Miyazawa et al., 1999; Matsumoto et al., 2001). Polyphenols have been detected in different tissues of mice and rats by high-performance liquid chromatography (HPLC), and their concentrations have been determined to be in the range of 30–3000 ng aglycone equivalents per gram tissue, depending on the dose administered, the type of tissue, and the time of tissue sampling (Manach et al., 2004). The authors also suggested that plasma concentrations are not directly correlated with concentrations in target tissues.

11.5.2.4 Metabolism and excretion

The metabolism of flavonoids is divided into two compartments. The first compartment consists of tissues such as the small intestine, liver, and kidneys, and the second compartment constitutes the colon. In the first compartment, biotransformation enzymes act upon flavonoids and their colonic metabolites (Hollman and Katan, 1998). The polar hydroxyl groups of flavonoids and their colonic metabolites are conjugated with glucuronic acid, sulfate, or glycine (Hollman and Katan, 1998). In addition, *O*-methylation of flavonoids, as well as deglycosylation of glycosides (Day et al., 2001) also occurs in the small intestine.

In the second compartment, which is the colon, microorganisms participate in the metabolism of flavonoids into phenolic acids (Olthof et al., 2003). Microorganisms degrade the flavonoid molecule by splitting the heterocyclic oxygen containing ring. Hollman and Katan (1998) have shown three types of ring fission products based on animal experiments. Flavonols are degraded to phenylacetic acids and phenylpropionic acids, catechins produce valerolactones and phenylpropionic acids, and flavones and flavanones produce phenylpropionic acids. Besides this, colonic bacteria produce glycosidases, glucuronidases, and sulfatases that can remove the sugar moieties, glucuronic acids, and sulfates, respectively, from the flavonoid conjugates (Scheline, 1973). All of the degradation products are subsequently absorbed and excreted via two routes. The larger flavonoid conjugates are excreted into the bile whereas the small conjugates are excreted through the urine (Manach et al., 2004).

11.6 GRAPES AND RED WINE AS A DIETARY SOURCE OF POLYPHENOLS

Grapes and wine have been a part of human culture for many years, serving dietary as well as socio-religious occasions (Soleas et al., 1997). The chemical composition of wine is profoundly influenced by enological techniques, the grape cultivar from which it originates, and climactic factors. Red wine contains up to 2.5 g/L of polyphenols and polyphenolic acids. The major compounds include flavonoids (up to 1.7 g/L) such as catechins, flavonols, anthocyanins, and oligomers (procyanidins) and polymers (tannins) of catechins. There are also nonflavonoids present up to 0.8 g/L, such as: phenolic acids, cinnamic acid, hydroxycinnamic acids, and stilbenes. Table 11.1 gives the general phenolics composition of red wines (German and Walzem, 2000). Furthermore, wines that are aged in oak barrels also contain simple phenolic components like vanillin, vanillic acid, syringaldehyde, syringic acid, gallic acid, ellagic acid, and so on, which are transferred into the wine from oak during oxidative aging of wines (Del Alamo Sanza et al., 2004). Although their bioavailability remains to be fully established, red wine provides a more favorable food matrix than fruits and vegetables, the alternative dietary source of flavonoids in humans. The polymeric and glycosidic forms of flavonoids in fruits and vegetables may not be easily degraded by digestive juices, and their absorption may be limited due to their relative insolubility in aqeous media. Wine fermentation breaks down the aggregates to monomeric forms, and the alcohol maintains the stability of flavonoids in solution, most likely in the intestine as well (Soleas et al., 1997).

11.6.1 Health benefits of red wine

The health benefits of wine consumption and potential issues associated with excessive consumption have been discussed in several studies (Purohit, 1998; German and Walzem,

Table 11.1. Phenolic acid and polyphenol components of red wines

	Mean concentration (mg/L)
Component	Red wine
Nonflavonoids	240–500
Hydroxybenzoic acids	0–260
p-Hydroxybenzoic acid	20
Gallic acid	116
Total gallates	40
Salicylic acid	–
Syringic acid	5
Protocatechuric acid	88
Hydroxycinnamic acids	162
Cis/trans-Coutaric	20
Cis/trans-Caftaric	25
Caffeic acid[a]	8.5
Coumaric acid[a]	12.6
Ferulic acid[a]	19
Stilbenes	12.3
trans-Resveratrol	1.0
Flavonoids	750–1060
Flavonols	98
Quercetin	18.8
Myricetin	16.2
Kamempferol	18
Rutin	6.8
Flavanols	168
Catechin	89
Epicatechin	57.3
Procyanidins	171
Anthocyanins	281
Delphinidin 3-monoglucoside[b,c]	22
Cyanidin 3-monoglucoside[b,c]	20
Petunidin 3-monoglucoside[b,c]	18
Peonidin 3-monoglucoside[b,c]	32
Malvidin 3-monoglucoside[b,c]	93
Total phenolic acids and polyphenols	1200

[a] Also present as tartrate esters.
[b] Also present as a diester with acetate.
[c] Also present as a diester with p-coumarate. Adapted from German and Walzem (2000).

2000). There is a special interest in red wine due to its high phenolic content. Red wine contains >200 phenolic compounds. Red wine has been shown to raise high-density lipoprotein levels, increase antioxidant potential, improve endothelium-dependent vasodilatation, and inhibit platelet aggregation and adhesion (Hayek et al., 1997; Halpern et al., 1998). Red wine may also contain ellagitannin compounds from oak (in case of oak barrel aged wines), and these compounds have also been linked to several potential health benefits (Caderni et al., 1999; Caccetta et al., 2000). Furthermore, it is postulated that a positive association exists between consumption of red wine polyphenolics and coronary heart disease (CHD) (De Gaetano and Cerletti, 2001).

Besides offering protection against cardiovascular diseases, grape and wine polyphenols have been of great interest in recent studies on cancer prevention (Singletary et al., 2003; Jacob et al., 2008). Polyphenols have been known to exhibit anticarcinogenic properties

through their ability to act as antioxidants, anti-inflammatory agents, and inducers of apoptosis and modulators of signal transduction pathways (Pietta et al., 1996; Eng et al., 2001; Ahn et al., 2003; O'Prey et al., 2003). Breast cancer is a hormone-dependent cancer, and since some of the polyphenols such as isoflavones act as phytoestrogens, they may positively or negatively influence breast cancer cell proliferation depending on several factors such as concentration, type of polyphenols, cell type, and so on. Therefore, understanding the biochemical and molecular mechanisms of action of grape and wine polyphenols against breast cancer is of great interest.

During an investigation exploring the effects of flavonoids on the growth and proliferation of human mammary cells and breast cancer cells, pure flavonoids such as catechin, quercetin, and naringenin were observed to inhibit the growth of normal cells in comparison to MCF-7, an ER positive breast cancer cell line. A similar response was also observed with the total polyphenols extracted from Merlot wine (Hakimuddin et al., 2004). Further fractionation of the wine polyphenols enabled the separation of a hydrophobic polyphenol fraction (red wine polyphenol fraction, RWPF, eluted from a C18 Sep-pak column using methanol: water (80 : 20 v/v) that showed relatively more inhibition of the cancer cells than normal cells (Figs. 11.1 and 11.2). By using a cell viability kit containing calcein-AM and ethidium homodimer, dead cells could be differentiated from live cells by the red fluorescence in the nucleus. The normal human mammary epithelial cells did not show cytotoxicity against red wine polyphenol fraction and grew normally even up to 72 hours after treatment. By contrast, the MCF-7 cells showed enhanced cytotoxicity after 24 hours of culture in the presence of the wine polyphenol fraction (Fig. 11.2) having lost cytoplasmic contents and becoming spherical.

Further studies were conducted to understand the mechanisms behind this cytotoxicity. MCF-7 cells and MCF-10 cells (spontaneously immortalized normal cells) were exposed to the red wine polyphenol fraction under *in vitro* conditions. The rapid damage to the MCF-7 after polyphenol treatment was indicative of major biochemical/molecular changes (Hakimuddin et al., 2006). To evaluate the potential disruption of calcium signal transduction system after treatment with polyphenols, changes in the levels of cytosolic calcium were estimated after polyphenol treatment. The cells were labeled with a calcium concentration sensitive fluorescent indicator Calcium Green 2 and examined under a confocal microscope. Within 8–12 minutes of RWPF treatment, an increase in fluorescence among several treated cells could be noticed suggestive of an increase in cytosolic calcium (Fig. 11.3A, top panel). After 24 minutes, the calcium green fluorescence disappeared. By contrast to MCF-7, the MCF-10 cells maintained a similar level of fluorescence after RWPF treatment (Hakimuddin et al., 2006) indicating the absence of an increase in cytosolic calcium.

The increase in fluorescence observed in MCF-7 cells through calcium release could be quantified by monitoring the changes by confocal microscopy and comparing it to that of calcium standards. After RWPF treatment, calcium levels increased to varying degrees in MCF-7 cells (Fig. 11.3C). The resting calcium levels varied between 20–160 nM in MCF-7 cells. After treatment with RWPF, the cytosolic calcium levels increased in general by 50–100%. The rapid drop in fluorescence, potentially due to mitochondrial sequestration or leakage of calcium can be seen after 24 minutes (Fig. 11.3C). There were no major changes in the fluorescence signals from MCF 10A cells, which remained nearly steady, suggesting that these normal cells resisted any changes in cytosolic calcium that were induced by RWPF treatment (Fig. 11.3D). Thus the RWPF effect was mediated through calcium.

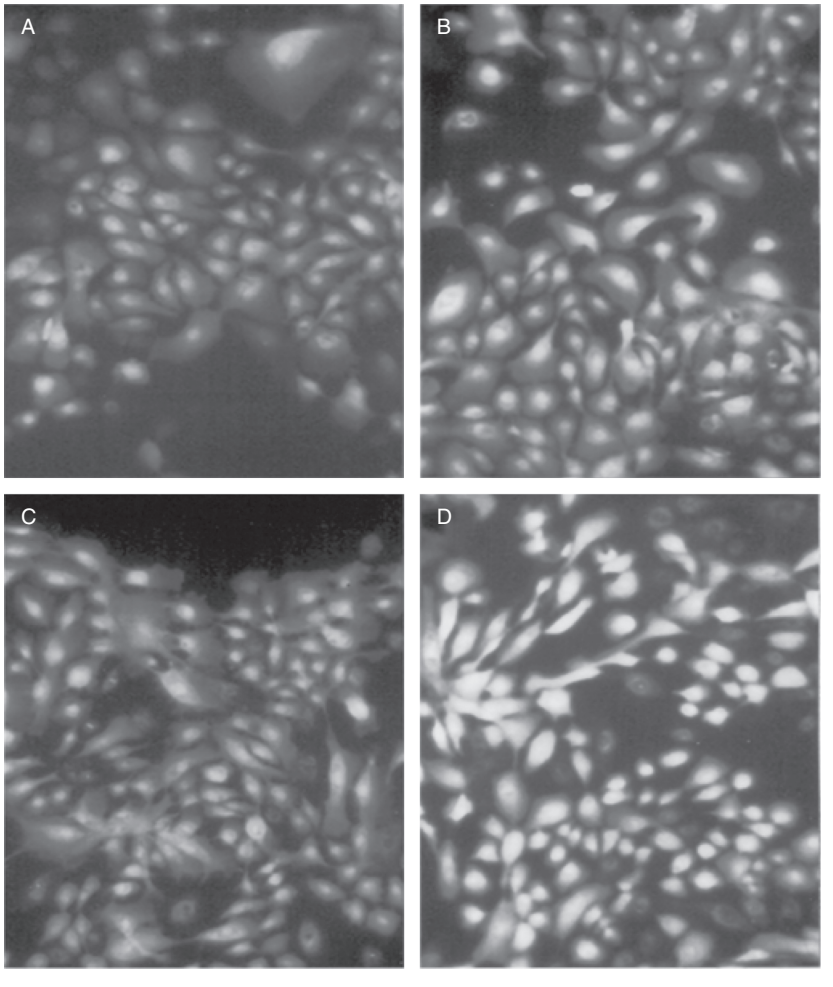


Figure 11.1. Effect of red wine polyphenols (Merlot, Fraction eluted with 80% methanol from a Sep-Pak cartridge) on the cell viability of normal human mammary cells. The cells were incubated in the presence of 50 µg polyphenols for 0 hour (A), 2.5 hours (B), 5 hours (C), and 24 hours (D). Reproduced with permission from Hakimuddin et al., 2004, *Breast Cancer Research and Treatment*, 85, 65–79.

Ultrastructural analyses of the cells were conducted to evaluate changes in subcellular structure that may have occurred after RWPF treatment. Within 3 hours of exposure to the polyphenols, the MCF-7 cells had lost their plasma membrane integrity, showed extensive membrane vesiculation within the cytoplasm, and nucleus that was reduced in size. After 12 hours of culture, the MCF-7 cells showed severe damage to the cell (Fig. 11.4A) as visible from the total damage to the internal membrane structure and organization. By contrast, MCF 10A cells did not show any disorganization and rupture of the membrane organization (Fig. 11.4B). The deleterious changes that occurred within the cell appeared to be resulting from necrosis rather than a programmed cell death (apoptosis), as there were no changes observed in caspase activity as well as DNA laddering. Calmodulin activity was estimated in untreated and RWPF treated cells by cAMP phosphodiesterase promotion, and there was no significant change in the activity after polyphenol treatment. Calmodulin

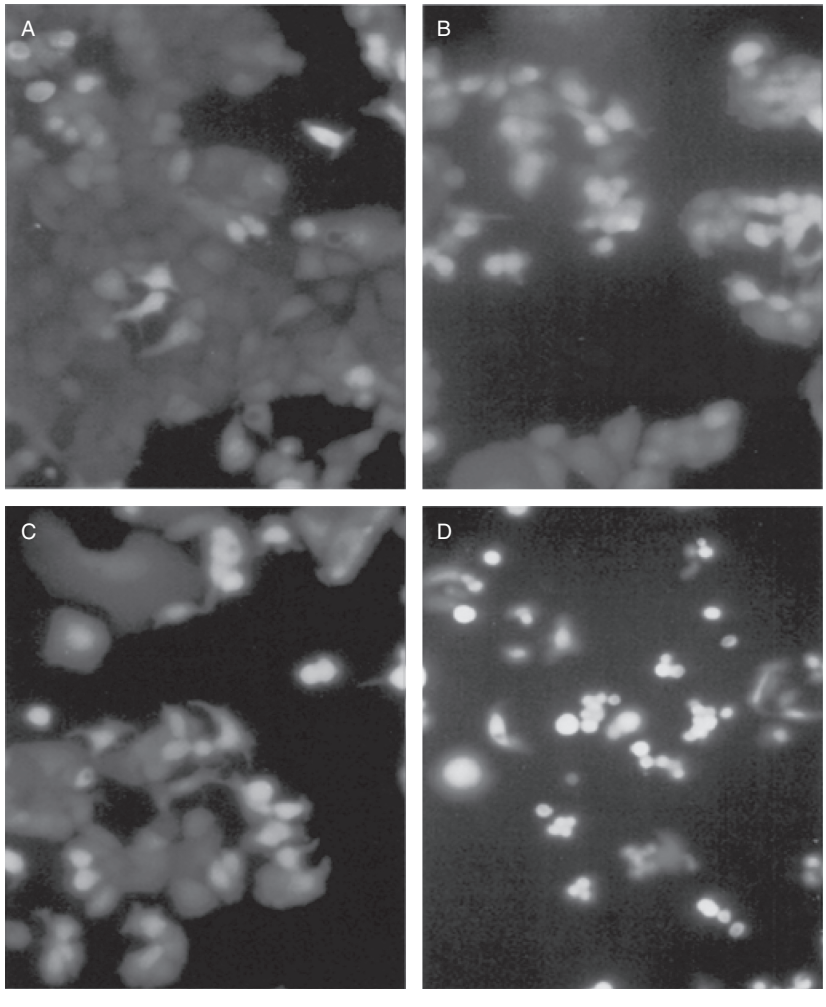
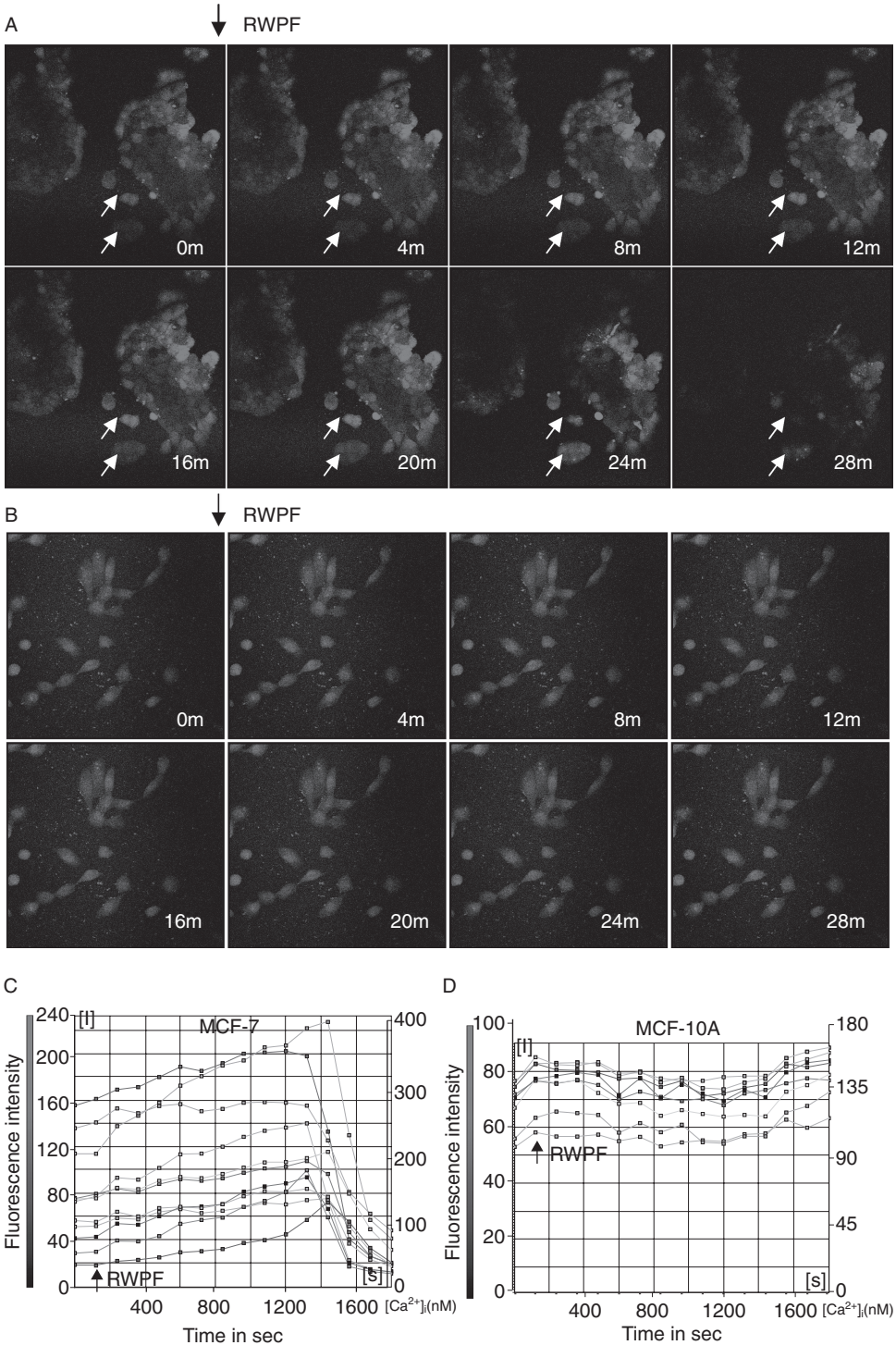


Figure 11.2. Effect of red wine polyphenols (Merlot, Fraction eluted with 80% methanol from a Sep-Pak cartridge) on the cell viability of MCF-7 breast cancer cells. The cells were incubated in the presence of 50 μg polyphenols for 0 hour (A), 2.5 hours (B), 5 hours (C), and 24 hours (D). Note the reduction in size and spherical structure of cells that are losing viability. Reproduced with permission from Hakimuddin et al., 2004, *Breast Cancer Research and Treatment*, 85, 65–79.

Figure 11.3. (A,B) Intracellular Ca^{2+} changes after RWPF treatment. Intracellular Ca^{2+} levels were measured in live cells via confocal laser scanning microscopy using the Ca^{2+} indicator dye Calcium Green-2. MCF-7 and MCF-10A cells were loaded with Calcium Green-2. Red wine polyphenols (RWPF) (50 μg) was added to the cells after basal images were recorded. Images of MCF-7 (Fig. 11.1A) and MCF-10A (Fig. 11.1B) cells collected every 4 minutes are shown. The number in the lower right corner of each image represents the time (minute) after addition of RWPF. Reproduced with permission from Hakimuddin et al., 2006. *Journal of Agricultural and Food Chemsitry*, 54, 7912–7923. (C,D) Changes in fluorescence intensity and corresponding Ca^{2+} levels in cells over time after RWPF treatment of MCF-7 (Fig. 11.1C) and MCF-10A cells (Fig. 11.1D) as shown in Figure 11.1A,B. Images were collected every 2 minutes for 30 minutes as indicated. Each line represents the change in Calcium Green-2 fluorescent emission of an individual cell over time. Calcium levels were calculated based on calibration using a standard of calcium at concentrations ranging from 0–400 nM. Increase in cytosolic calcium levels of MCF-7 cells after RWPF treatment varied between 50–100% of the basal levels. Reproduced with permission from Hakimuddin et al., 2006. *Journal of Agricultural and Food Chemsitry*, 54, 7912–7923.


A
RWPF
0m
4m
8m
12m
16m
20m
24m
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B
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C
MCF-7
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Fluorescence intensity
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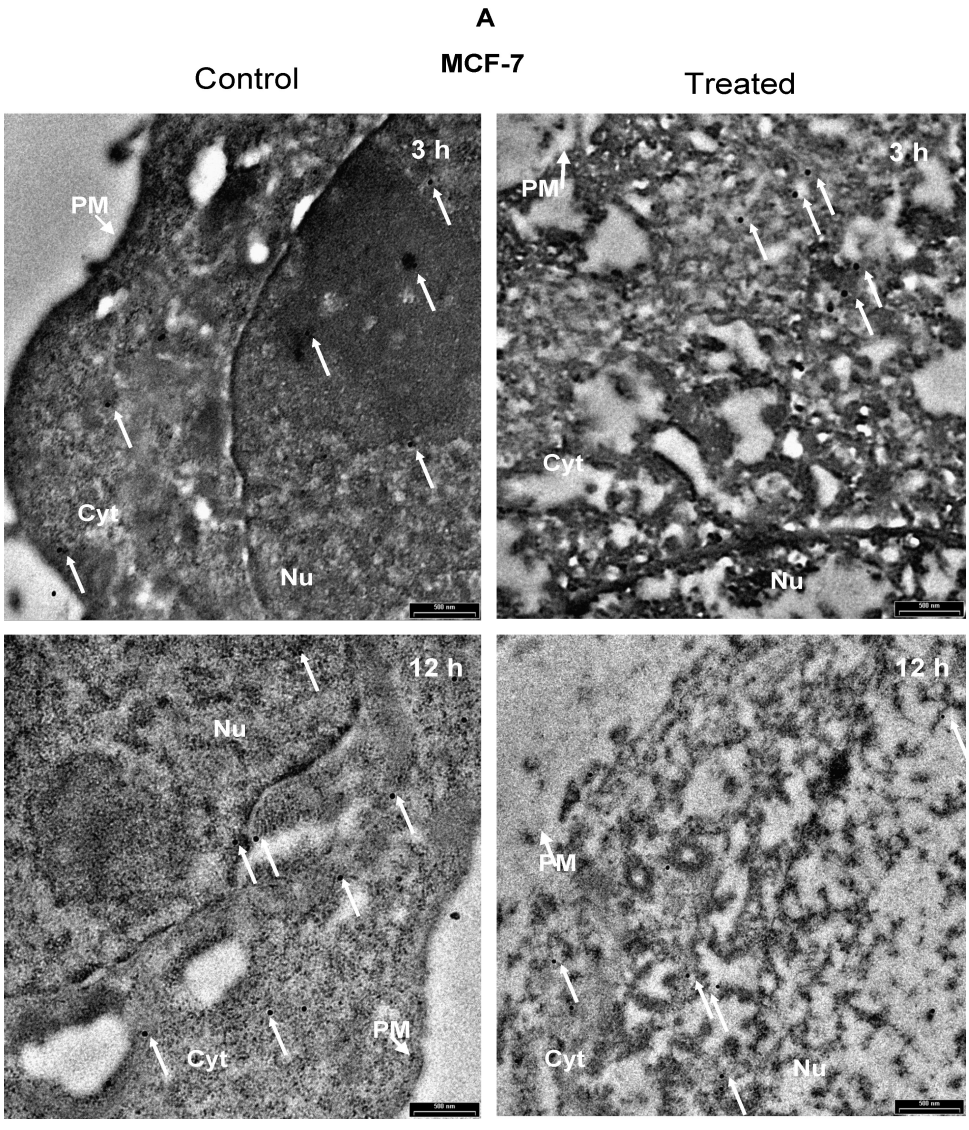

Figure 11.4. Transmission Electron Micrographs of MCF-7 and MCF-10A cells immunolabeled with monoclonal anti-calmodulin antibody and secondary antibody (goat-antimouse IgG) labelled with 20 nm gold particles. (A) Ultrastructure and immunolocalization of calmodulin in MCF-7 cells untreated (3 hours, 12 hours, left panel) and treated with 50 µg RWPF (3 hours, 12 hours, right panel). Calmodulin labeled with the gold appear as dark dots as shown by the arrows. Plasma membrane (PM), cytoplasm (Cy), and Nucleus (Nu) are shown. Extensive vacuolation in the cytoplasm of treated MCF-7 cells are visible as early as three hours (Vac). After 12 hours of treatment, membrane integrity was severely disrupted and the dissolution of cytoplasmic and nuclear membrane was apparent. (B) Ultrastructure and immunolocalization of calmodulin in MCF-10A cells untreated (3 hours, 12 hours, left panel) and treated (3 hours, 12 hours, right panel) with RWPF. Calmodulin bound to antibody-gold conjugates are shown by arrows. The plasma membrane (PM), cytoplasm (Cy) and nucleus (Nu) are shown. The figures are representative of several such cells showing similar structural features. The bar represents 500 nm. Reproduced with permission from Hakimuddin et al., 2006. *Journal of Agricultural and Food Chemsitry*, 54, 7912–7923.

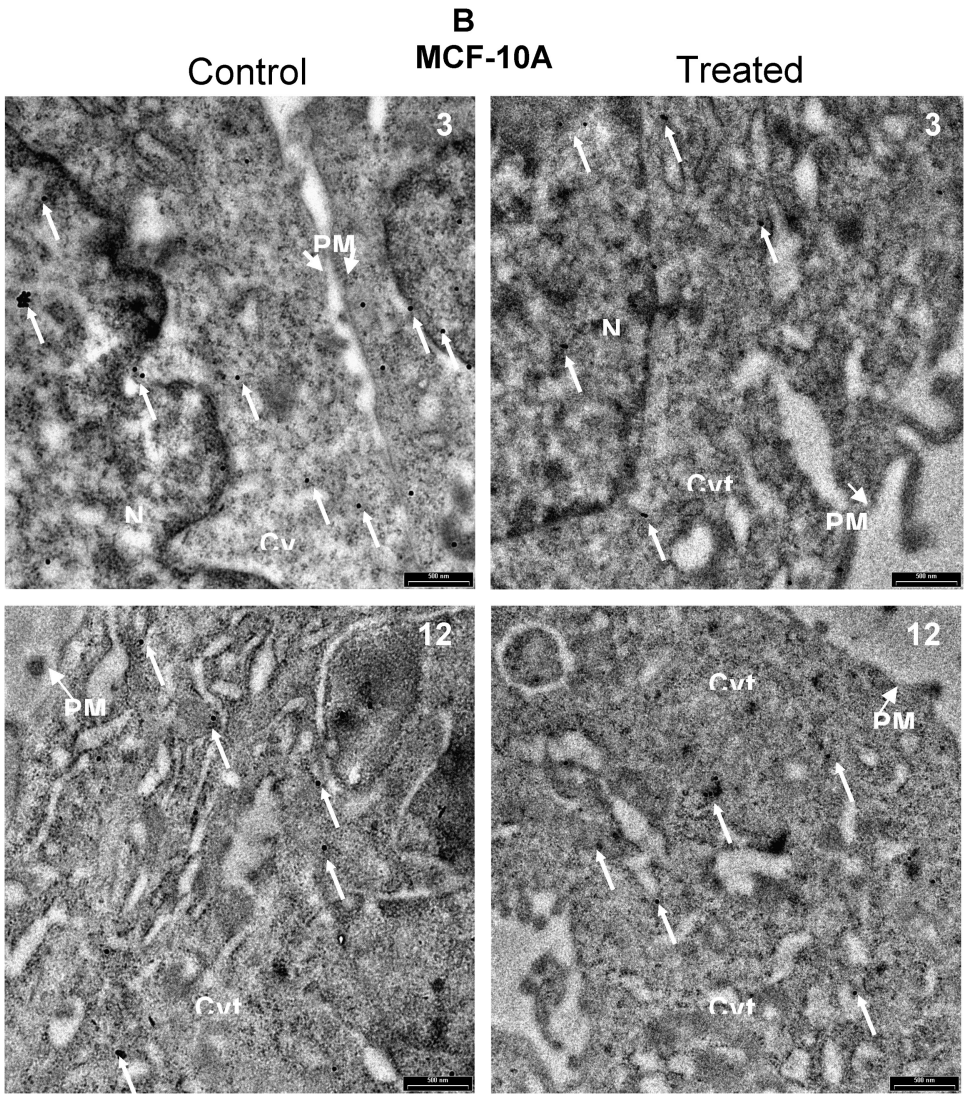

Figure 11.4. (*continued*)

was also localized by immunoelectron microscopy using rabbit anti-calmodulin antibody and detection using 20 nm gold loaded secondary antibody. The location of gold particles indicating calmodulin can be seen in Figure 11.4A,B. Again, there were no major changes in the number of calmodulin molecules in the cell after RWPF treatment. This suggested that changes in calmodulin expression or activity may not be a significant event in polyphenol-mediated events.

The effect of RWPF in inhibition of cell proliferation may also be mediated through a block in cell division. Such cell cycle arrest could occur through blocks at critical steps in cell division as explained earlier. Even though there were no measurable changes in calmodulin activity, localized and specific inhibition of cell cycle at steps regulated by

calcium/calmodulin may be an effective means of arresting cell proliferation. MCF-7 and MCF-10A cells at 60–70% confluence were treated with RWPF for 48 hours, isolated and loaded with propidium iodide. The cells were subjected to fluorescence activated cell sorting by which the population of cells at various stages of division can be separated and quantified by their fluorescence (Fig. 11.5A,B). In MCF-7 cells, treatment with RWPF resulted in an increase in the number of cells in the "G2/M" phase (25%) with a concomitant decrease in the S phase. This showed that the RWPF treatment caused a cell cycle arrest at the G2/M phase, preventing the cell cycle from proceeding from the M phase into the DNA synthesis phase as a prelude to the next cell division. The number of cells in the "S" phase remained almost similar in untreated and treated cells. By contrast to MCF-7 cells, the MCF-10 cells exposed to RWPF did not show a G2/M arrest as the proportion of cells in G1, S and G2/M phases remained similar in both untreated and treated cells. These results showed that by comparison to cancer cells, normal cells are not affected by the presence of polyphenols, and this may serve as a cancer prevention strategy.

11.6.2 Modulation of signaling pathways by flavonoids

Most of the biological actions of flavonoids have been attributed to their antioxidant properties (Manthey, 2000; Coşkun et al., 2004), either through their reducing capacities or through their possible effects on intracellular redox status. However, several studies have hypothesized that in addition to their antioxidant potential, flavonoids exhibit a diverse range of effects on various signal transduction pathways regulating cell growth, cell cycle control, or apoptosis (Ferriola et al., 1989; Dowd et al., 1991; Singhal et al., 1995; De Azevedo et al., 1996). Inhibitory or stimulatory actions at these pathways affect cellular function by altering the phosphorylation state of target molecules and by modulating gene expression. The flavonoids silymarin and apigenin have been shown to inhibit activation of the MAP kinase pathway by EGFR (Wenzel et al., 2001) and Ras (Kuo and Yang, 1995). A major tea polyphenol, epigallocatechin-3-gallate (EGCG), inhibits MAPKs (Chung et al., 2001), cyclin-dependent kinases, and NF-κB (F. Yang et al., 2001).

There is considerable evidence supporting the possibility of modulating the calcium/calmodulin pathway in the treatment of cancer especially breast cancer (Dai et al., 2002), since calcium homeostasis and signaling is important in breast cell physiology. Due to the duality in calcium signaling, the functional consequences of Ca^{2+} entry appear to be determined by a threshold, with cell proliferation possibly being stimulated below a threshold and cell death being stimulated above this (Sergeev, 2004). The antiestrogen compound tamoxifen, which is an established agent used in the endocrine treatment of breast cancer, can induce an increase in $[Ca^{2+}]_i$ in ZR-75-1 breast cancer cells and can also reduce cell viability (Chang et al., 2002). This is mediated by the release of Ca^{2+} from the ER and the subsequent induction of capacitative Ca^{2+} entry (Chang et al., 2002). Tamoxifen has also been shown to induce significant increases in $[Ca^{2+}]$i, causing cytotoxicity in human prostate cancer cells and rat glioma cells.

Flavonoids also have different effects on the cell cycle. They cause a G1 or G2 cell cycle arrest in different contexts, and this is associated with inhibition of cell cycle kinases, CDK2, or CDK1. For for example, quercetin blocks the cell cycle at the G1/S transition in gastric cancer cells and leukemic cells (Yoshida et al., 1992), whereas it causes a G2/M block in breast and laryngeal cancer cell lines (Ferrandina et al., 1998). Genistein induces both G1 and G2 blocks in BALB/c 3T3 fibroblasts or mouse melanoma cells (Kuzumaki et al., 1998). In a recent study, specific polyphenol fractions from cranberry have been

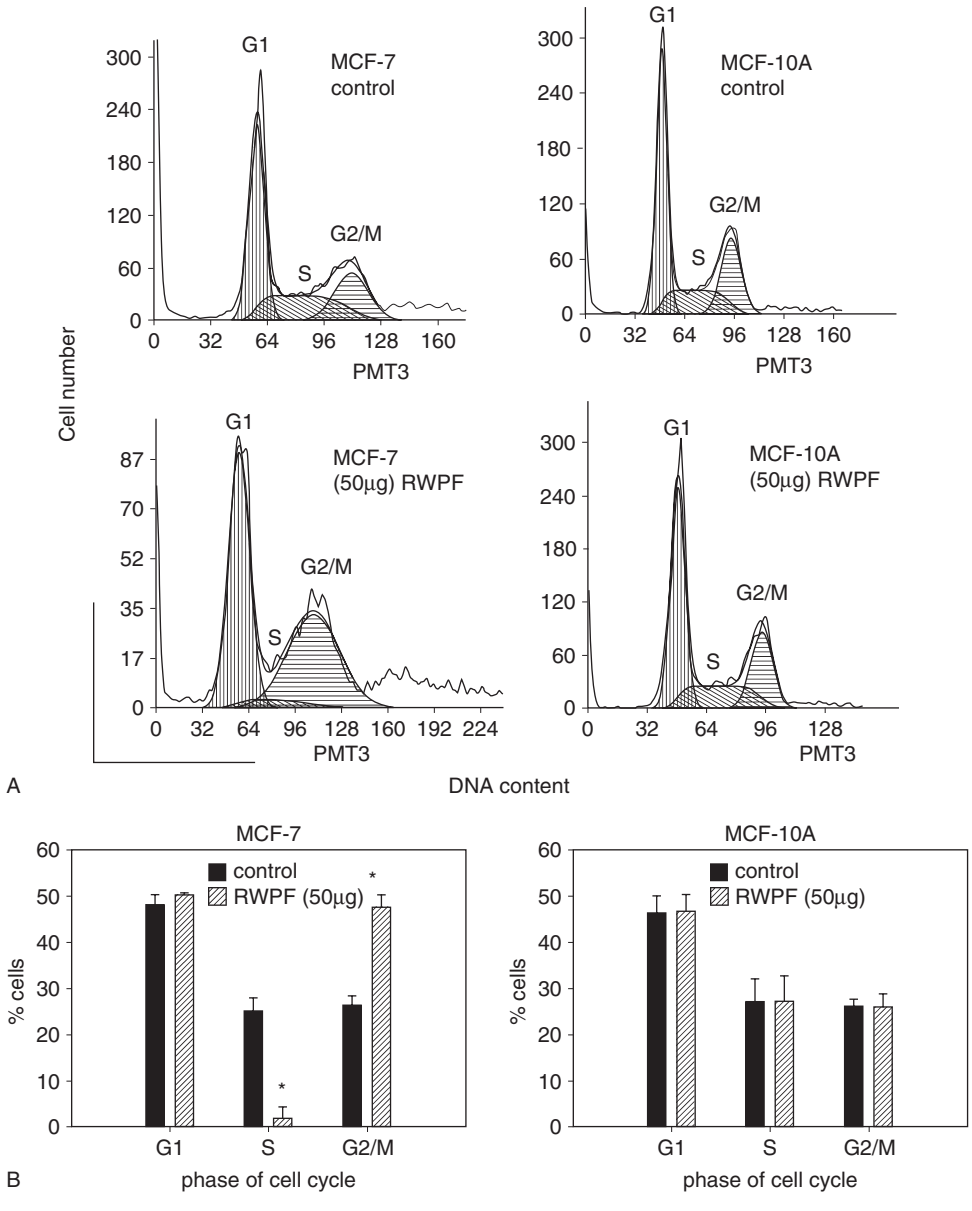

Figure 11.5. A, B. Effect of RWPF treatment on the cell cycle. Cell cycle profiles of MCF-7 and MCF-10A cells (60–70% confluence) after treatment with 50μg RWPF for 48 hours. The cells were labelled with propidium iodide and subjected to Fluorescence-activated cell sorter (FACS) analysis. Panel A shows the distribution of cells in G1, S and G2/M phases in MCF-7 and MCF-10A cells that are untreated and subjected to RWPF treatment. Quantitative estimation of the % cells in G1, S, and G2/M is shown in Panel B. MCF-7 cells showed a dramatic increase in G2/M phase cells from 26.4% to 47.6%, indicating a G2/M block and a decrease in S phase cells from 25.3% to 1.9%. The values are mean ± SE from three independent evaluations. Statistical analysis was conducted using unpaired *t*-test and significant differences at $P < 0.05$ versus the controls are marked by asterisks. Reproduced with permission from Hakimuddin et al., 2006. *Journal of Agricultural and Food Chemsitry*, 54, 7912–7923.

shown to induce apoptosis in several human breast cancer cell lines through blocking cell cycle progression (Ferguson et al., 2004a). Three dietary flavonoids, kaempferol, apigenin, and luteolin were shown to cause induction of the ATR-p38-GADD45 pathway which is a stress kinase pathway controlling G2 cell cycle checkpoint, resulting in G2 arrest.

11.7 GENETIC APPROACH: IDENTIFICATION OF FLAVONOID MEDIATED MOLECULAR TARGETS

Several researchers have studied the molecular mechanisms of flavonoid action *in vitro* and *in vivo* using a variety of proteomic and/or genomic approaches. However, most of the methods are time-consuming and enable the analysis of only a few genes or proteins. Array-based technology is one of the latest methods that allow one to study gene expression changes on a larger scale in a shorter time period. This is a more comprehensive approach to studying pathways and mechanisms that are involved in cancer prevention. Guo (2005) has shown that EGCG treatment induces changes in expression of a large number of genes involved in proliferation control, cell cycle control, and apoptosis, in different cancer cell lines by greater than twofold. Banerjee et al. (2002) showed that resveratrol suppression of DMBA-induced mammary carcinogenesis was correlated with the downregulation of NF-κB, COX-2, and matrix metalloprotease-9 (MMP-9) expression. The effect of quercetin on the expression of 4000 human genes in Caco-2 cells was studied by Erk et al. (2005) in order to elucidate possible mechanisms involved in its mode of action. Their results indicated that quercetin (5 μM) downregulated the expression of cell cycle genes (e.g., CDC6 [cyclin-dependent kinase 6], CDK4 [cyclin-dependent kinase 4], and cyclin D1), and upregulated the expression of several tumor suppressor genes. This correlated with the downregulation of cell proliferation and induction of cell cycle arrest in Caco-2 cells. In addition, quercetin also modulated genes involved in signal transduction pathways such as the beta catenin/T-cell factor (TCF) signaling and MAPK signaling (Erk et al., 2005).

Epidemiological evidence shows a strong association between consumption of fruits and vegetables and cancer prevention (reviewed by Block et al., 1992). However, epidemiological studies suggesting a positive correlation between polyphenols and cancer prevention are largely unavailable or inconclusive. Several preclinical animal studies have demonstrated the chemopreventive activity of flavonoids such as green tea catechins, curcumin, anthocyanins, quercetin, and silibinin. For example, (-) epigallocatechin-3-gallate given as an intraperitoneal injection at a concentration of 1 mg rapidly inhibited MCF-7 mammary tumor growth in nude mice (Liao et al., 1995). Also, green tea extract has been shown to inhibit the growth of MDA-MB231 xenografts in *scid* mice by greater than ten fold at day 35, at a concentration of 2.5 g/L administered through water (Sartippour et al., 2001). Ferguson et al. (2004b) demonstrated that an acidified methanolic extract (Fr6) and a proanthocyanin fraction (PACs) from cranberry are able to inhibit U87 (human glioblastoma) tumor cell growth *in vivo*, suggesting that they have a potential role in cancer treatment. They showed that immuno-compromised male mice injected intraperitoneally with Fr6 (250 mg/kg) or PACs (100 mg/kg) every 2nd day up to day 21 significantly inhibited tumor growth (average tumor sizes up to 60% smaller, $P < 0.05$). Red wine polyphenols (50 mg/kg) administered with the diet to F344 rats for 16 weeks inhibited colon carcinogenesis induced by azoxymethane or dimethylhydrazine (Dolara et al., 2004, 2005). In another study, Chen et al. (1998) demonstrated the breast cancer-preventive action of grape juice

with a nude mouse model using MCF-7aro, an aromatase-transfected MCF-7 cell line. It was found that in mice fed by gavage with 0.5 mL of grape juice/day for 5 weeks, the tumor size was reduced by 70% compared to the tumor size in the animals that were not fed grape juice.

The possibility that grape and wine polyphenols could act as cancer chemopreventive agents was investigated using an athymic mouse model transplanted with the ER-negative MDA-MB231 breast cancer cells, and evaluating their ability to modulate gene expression in developing tumors. Groups of mice were fed with a modified AIN 93G diet with the experimental groups receiving 100 mg/kg body weight equivalent of polyphenols (polyphenols from Merlot grapes, Merlot wine, and a fraction eluted between 60% and 80% methanol from a C18 Sep-Pak column) by gavage thrice per week. Following 1 week of acclimation and another week of polyphenol supplementation, MDA-MB 231 cells were transplanted and the growth patterns of the tumors monitored. After 33 days of tumor growth, the animals were euthanized, the tumors isolated, and gene expression profiles analyzed using signal transduction and cell cycle arrays.

Polyphenol components in Merlot grape, wine, and 60–80% wine extract significantly inhibited ER-negative MDA-MB231 tumor cell proliferation in mice (Fig. 11.6). The degree of tumor growth inhibition by grape polyphenols was more than that of the wine polyphenols and the 60–80% wine fraction. Since the total polyphenol concentration used in all treatments were similar, the differences in the degree of inhibition may be related to the compositional differences of the grape and wine extracts and the metabolism of the respective polyphenol components in the body. Liquid chromatography–mass spectrometry (LC-MS) analysis showed that the grape polyphenol fraction contained primarily anthocyanins, while the 60–80% fraction contained relatively more hydrophobic anthocyanins such as acetoylated and coumaroylated anthocyanins. The grape polyphenol extract was enriched

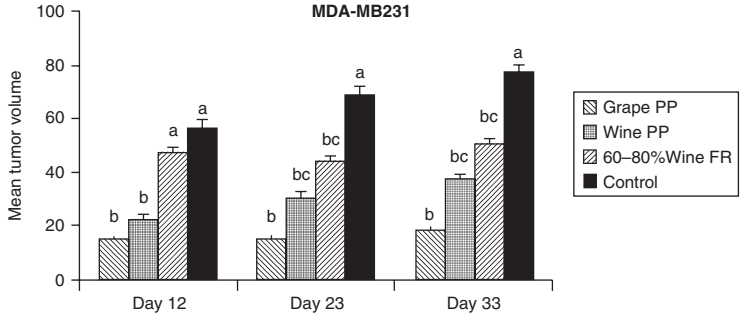

Figure 11.6. Effect of grape and wine polyphenols on the growth of MDA-MB231 breast cancer xenograffs. Athymic nude mice nu/nu (eight per group) were injected with 3×10^6 MDA-MB231 cells subcutaneously in the right flank. Experimental mice were gavaged thrice weekly with 100 mg/kg body weight of grape (Grape PP) and wine polyphenols (Wine PP; 60–80%Wine FR), and control mice were gavaged with water. Grape and wine polyphenols were purified through a Sep-Pak C18 cartridge. The grape polyphenols were eluted with 100% methanol. The wine polyphenols were either eluted with 100% methanol (wine PP) or successively eluted with methanol: water mixtures. The polyphenol fraction eluting between 60% and 80% methanol was used (60–80%wine FR). Tumor size was measured in three dimensions with callipers twice weekly and expressed as volume (mm^3). Values are means ± SD of tumor volumes. The histograms with different alphabets indicate values that are significantly different at $P < 0.05$. Reproduced with permission from Hakimuddin et al., 2008, *Nutrition Research*, 28, 702–713.

Table 11.2. Table showing signal transduction genes modulated by grape polyphenols in MDA-MB231 tumors. In general, genes that were downregulated by greater than 3-fold as compared to the control are shown

Downregulated genes	Fold-change	*P*-Value
CDK2	–2.93	0.029
FAS	–3.73	0.011
IGFBP3	–5.09	0.290
IL1A	–3.48	0.197
IL8	–3.73	0.191
LEF1	–1.68	0.040
MMP7	–8.87	0.091
ODC1	–3.36	0.095
PRKCE	–1.68	0.012
PRKCA	–3.13	0.108
PTGS2	–8.57	0.006
RBP1	–12.12	0.105
TFRC	–3.03	0.194
TMEPAI	–6.73	0.110
VEGF	–3.86	0.092

Values are means of three replicates from three different tumors. Reproduced with permission from Hakimuddin et al. (2008), *Nutrition Research*, 28, 702–713.

in the glucosides, whereas these were present in lesser amounts in the wine polyphenol extract and in trace amounts in the 60–80% fraction (Hakimuddin et al., 2008). Whether these glucosides were responsible for the increased biological activity of the grape polyphenols against the growth of MDA-MB231 cells *in vivo* is not clear at this point.

Overall results indicated that most genes implicated in cell proliferation and cell cycle progression were downregulated by grape polyphenol treatment in MDA-MB231 tumors, though few genes were upregulated (Hakimuddin et al., 2008). The significant downregulation of the cytokine family of genes such as the interleukins (IL1A, IL4R, IL8-NFk-B pathway) and PTGS2 (prostaglandin synthase-PLC pathway) indicated that the inflammatory pathway may be involved in inhibition of tumors in grape and wine polyphenol-treated mice (Table 11.2). Downregulation of ODC1 (ornithine decarboxylase 1) and MMP7 (matrix metalloproteinase) (Sternlicht and Werb, 2001) may also have played a role in tumor inhibition. This is supported by studies which show that specific inhibition of ODC enzymatic activity and downregulation of MMP results in chemoprevention (Weeks et al., 1982), and reduced tumor burden or metastasis (Sternlicht and Werb, 2001). Genes downregulated in the 60–80% fraction treated MDA-MB231 tumors were mostly the estrogen responsive genes, such as FOXA2 (forkhead box A2), HSPB1 (heat shock 27 kDa protein 1), and NRIP1 (nuclear receptor interacting protein 1). These genes are known to modulate the transcriptional activity of the ER (Carroll et al., 2005) and their function may not be as critical in ER negative cells as MDA-MB 231. Consequently, polyphenols from wine and the 60–80% fraction were less effective in inhibiting the growth of ER negative MDA-MB231 tumor growth in mice.

Results from the animal experiments suggest that polyphenols in their natural form may show beneficial activity due to a combinatorial effect of different components and their action on multiple targets. In conclusion, this study suggests that grape and wine polyphenols may exert a broad range of effects at gene expression level on breast cancer cells.

Inhibition of signaling pathways such as PLC and NF-κB pathway may be involved in polyphenol-mediated tumor growth inhibition in MDA-MB231 breast cancer cells. In addition, modulation of genes in the cell cycle such as cyclins, cyclin-dependent kinases, and cell cycle arrest [(GADD45A, growth arrest and DNA-damage-inducible, alpha), CDKN1A (cyclin-dependent kinase inhibitor 1A (p21, Cip1), (TP53, tumor protein p53 (Li–Fraumeni syndrome)] indicates that grape and wine polyphenols may influence cell proliferation *in vivo*. The study shows the importance of consuming food ingredients in their natural form and the beneficial effects it could have in preventing chronic diseases (Liu, 2004). Similar observations have been summarized in a recent review on the mechanism of cancer prevention by green and black tea polyphenols (Beltz et al., 2006). Tea polyphenols modulated the activity of key enzymes involved in cellular regulation that included MAP kinases and PKC. Modulation of transcription resulted in altered transcript levels of cyclins, several oncogenes and tumor suppressor genes. Enzymes associated with metastasis such as urokinase and matrix metalloproteinases were also inhibited. Tea polyphenols also inhibited angiogenesis by reducing the levels of vascular endothelial growth factor and phosphorylation of receptors. Thus, polyphenols from different dietary sources appear to exert their effects through common pathways.

11.8 ESTROGEN METABOLISM, BREAST CANCER, AND FLAVONOIDS

Proliferation of breast cancer is mostly estrogen-dependent, and polyphenols may influence estrogen metabolism in the body due to their ability to inhibit the enzymes responsible for detoxification of hormones (Zhai et al., 1998). Furthermore, polyphenols have been shown to act as estrogen agonists, antagonists, or inhibitors of biosynthesis in different contexts (Wang et al., 1996; Eng et al., 2001; Schmitt and Stopper, 2001; Woude et al., 2005). These factors, as well as diet and lifestyle (Dai et al., 2001; Sowers et al., 2006; Fuhrman et al., 2009), may play a role in determining the potential for the development of breast cancer.

Long-term exposure to higher levels of estrogen has been associated with breast cancer because of its ability to cause cell proliferation (Hankinson et al., 1995, 1998). Estrogen has been shown to cause an increased expression of oncogenes such as *c-myc*, *ras*, and *bcl-2* in animals and cultured cells, resulting in tumorigenesis and abrupt cellular proliferation (Dubik and Shiu, 1992; Pethe and Shekhar, 1999; Perillo et al., 2000). The effects of estrogen are associated largely with the way in which it is metabolized. Estrogen metabolism depends on three factors: a woman's genetic makeup, lifestyle and diet, and environment (Taioli et al., 1996; Coker et al., 1997). In premenopausal women, the ovaries produce the estrogen estradiol (E2), which is converted into estrone (E1). Prior to conjugation and excretion into the bile or urine, estrone and estradiol are hydroxylated at either the carbon-16 or the carbon-2 positions by cytochrome P-450 dependent enzymes. Prospective and case-control studies suggest that women with higher urinary 2-OHE/16-OHE ratios are less likely to be diagnosed with breast cancer (Kabat et al., 1997; Ho et al., 1998; Muti et al., 2000). This is because the 16α-hydroxyestrone (16-OHE) metabolite has a higher affinity for the ER and promotes cell proliferation (Osborne et al., 1993) while the 2-hydroxyestrone (2-OHE) metabolite has a low affinity for the ER and reduces tumor growth and angiogenesis (Schumacher and Neuhaus, 2001).

Dietary modifications can shift estrogen metabolism predominantly through the C2 hydroxylation metabolic pathway. Increasing the amount of cruciferous vegetables in the

diet has been shown to increase the C2:C16 ratio. Consumption of 10 g/day of *Brassica* vegetable was associated with a statistically significant increase in 2:16 ratio of 0.08 in urine samples (Fowke et al., 2000). Isoflavones have also been shown to increase the C2:C16 ratio in urine (Guerra et al., 2000; Lu et al., 2000).

11.9 POLYPHENOLS AND ESTROGEN SIGNALING

The effects of estrogen are transduced through the classic nuclear receptors called as estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β), which belong to the superfamily of nuclear receptors. Estrogen signaling through ER occurs through three pathways. First is the classical pathway, where ligand-activated ER binds to DNA at estrogen-responsive elements (EREs) through its DNA binding domain and recruits coactivators and corepressors to the transcription site via AF-1 and AF-2 (activator function domains 1 and 2). Another mechanism is through the interaction of ER with other transcription factors such as AP-1, NF-κB, or SP-1 that in turn activate gene expression. This mechanism is called as cross talk (Stein and Yang, 1995; Kushner et al., 2000). A third mechanism by which estrogen and the ER affect gene expression is called the nongenomic pathway. In this pathway, estrogen binds to the ER localized in the membrane or cytoplasm that in turn activates signal transduction pathways in the cytosol. Through this mechanism, estrogen rapidly activates MAPKs and PKC (Tesarik and Mendoza, 1995; Kelly et al., 1999) that further leads to induction of genes downstream of these kinase cascades.

There is a great deal of interest in developing drugs to block the undesired activity of estrogens. Selective ER modulators (SERMs), a group of pharmacologically active ER ligands, have been developed and designed to mimic estrogen's beneficial actions and prevent unwanted effects in specific tissues (Park and Jordan, 2002; Jordan, 2004). Studies have shown that soy-derived isoflavonoids such as genistein, daidzein, and a flavonol such as kampferol, hamper cell proliferation *via* their binding to ERα and interfering with its rapid signaling (Birt et al., 2001; Chen et al., 2003; Hung, 2004). Stimulation of transcriptional activities by estrogen is mediated through ER-α, and ER-β inhibits the transcriptional activity of ER-α (Jensen et al., 2001; Sun et al., 2001; Weihua et al., 2002). In this context, Lee et al. (2005) showed that selenium exhibited cancer-preventive properties through differential regulation of ER. Selenium inhibited the expression of ER-α and increased the expression of ER-β in MCF-7 and MDA-MB231 cells, respectively. Similarly, Kuiper et al. (1998) established that the isoflavonoid genistein has agonist activity for both ER-α and ER-β, but genistein's affinity for ER-β is considerably greater, and shown to be 8.4 nM as compared to its affinity for ER-α (145 nM). Flavonoids have also been shown to have biphasic effects in MCF-7 cell death. Some studies suggested that the proliferating phase is due to estrogenic activity of flavonoids, while the cytotoxic phase is due to their antiestrogenic activity (So et al., 1997; Le Bail et al., 1998).

Results from the above studies suggest that the interaction of flavonoids with estrogen and its receptor is complex and needs to be studied in more detail at the molecular level. Also, tumor growth and progression *in vivo* is a result of several complex mechanisms regulated by the environment in which the tumor is growing and its interaction with the surrounding normal tissue. Therefore, multiple approaches should be adopted to study tumor growth and the mechanisms underlying their inhibition. By contrast to the effects of polyphenols on estrogen receptor negative tumor development (Hakimuddin et al., 2008), the effects on estrogen positive (MCF-7) tumor development were not as marked. The

reasons for this difference are not clear. The mice receiving MCF-7 cells were transplanted with 0.72 mg (one pellet per mouse, 90-day slow release) pellets of 17-β-estradiol before initiating the treatment, which is required for the growth of the transplanted MCF-7 cells. Perhaps, this amount may be too high and could have altered the response of tumors to polyphenols absorbed by the body [which is in the range of 0.5–1%; assuming 1% uptake from 2.5 mg polyphenols 3 times per week (100 mg/kg equivalent, an average mouse weighs 25 g), the potential bioavailability may be in the range of 75 μg/week). Perhaps this concentration may not be enough to suppress the effects of 8 μg/day release of estradiol. Further studies are needed to resolve these differences.

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12 Potato–Herb Synergies as Food Designs for Hyperglycemia and Hypertension Management

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12.1 INTRODUCTION

The incidence of type 2 diabetes is becoming a global epidemic, primarily due to dramatic changes in diet and lifestyle. Diabetes mellitus occurs due to high blood glucose levels and a body's inability to respond to high blood glucose, caused by defects in insulin secretion and insulin action (Schulze and Hu, 2005). Diabetes mellitus harms a significant amount of people worldwide, in both developed and developing nations. Type 2 diabetes develops when the cellular sensitivity to insulin signaling is reduced, and is known to comprise 90–95% of all diabetic cases globally (Schulze and Hu, 2005). Type 1 diabetes is caused by the complete deficiency of insulin secretion, which is due to the destruction of pancreatic beta cells. Type 2 diabetes is known to be caused by diet, lifestyle, and environmental risk factors, but for type 1 diabetes, there are only a few known risk factors that could cause the disease (Schulze and Hu, 2005). The adoption of a Western diet and risk factors such as obesity and the lack of physical activity are known to be linked with the high occurrence of type 2 diabetes (Van Dam et al., 2002).

The number of people suffering from type 2 diabetes is rising consistently worldwide. According to the World Health Organization there were 35 million people with type 2 diabetes in 1985, which increased to 220 million people in 2009 (http://www.afro.who.int/en/rdo/messages-and-press-statements/1954-message-of-the-regional-director-on-the-occasion-of-world-diabetes-day-2009.html). Due to such an increase in such a short period of time, we can assume that the epidemic nature of type 2 diabetes is due to environmental, diet, and lifestyle risk factors rather than genetic factors. Type 2 diabetes is associated with obesity, and the estimated number of people suffering from type 2 diabetes is predicted to rise to over 350 million by 2030 (http://whqlibdoc.who.int/trs/WHO_TRS_916.pdf).

Hypertension and associated cardiovascular diseases are a consequence of type 2 diabetes, and many people suffering from type 2 diabetes die of some sort of cardiovascular complication (Schulze and Hu, 2005). People suffering from type 2 diabetes have 2–6

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times higher risk of cardiovascular diseases than people without type 2 diabetes (Gaede et al., 2003). Cardiovascular diseases and microvascular and macrovascular complications associated with type 2 diabetes are known as leading causes of blindness, kidney failure, and amputations (Gaede et al., 2003; Schulze and Hu, 2005). The medical cost of treating diabetes and its associated illnesses is enormous and is constantly rising as the rate of diabetes mellitus cases rises. The medical cost was estimated to be over $132 billion in 2002 (Report from the American Diabetes Association, 2002).

Since there is no cure for the incidence of type 2 diabetes, raising awareness of changing diet and lifestyle is the best strategy to reduce the incidence of such illnesses. The progress achieved in the development of synthetic drugs for the therapy of type 2 diabetes is remarkable. However, the development of antidiabetic substances from natural sources for managing hyperglycemia and hypertension linked to type 2 diabetes is being pursued due to their low toxicity, potentially fewer side effects, and low cost for global use (Ahmad et al., 2009; Pinto and Shetty, 2010). These plant-based foods contain basic nutrients such as carbohydrates, protein, vitamins, minerals, dietary fibers, and more important bioactive compounds such as polyphenols and carotenoids (Montonen et al., 2004; Robert et al., 2006), which have specific structure–function benefits (Shetty et al., 2008; Pinto and Shetty, 2010). Consumption of these bioactive compound-enriched plant-based foods has the potential to prevent and lower the incidence of early stages of chronic illnesses, such as type 2 diabetes and cardiovascular diseases, and also to manage their late-stage micro- and macrovascular complications.

Plant-based foods used for medicinal therapies have been utilized since ancient times for the treatment of common illnesses. Various plant foods contain antimetabolites, which prevent the oxidation pathway of fatty acids (Ahmad et al., 2009), serving as a potential for managing hyperglycemia. Inclusion of diets rich in fruits and vegetables compared with diets favoring red meat and sweets, independent of physical activity and family history of diabetes, presents a lower risk for type 2 diabetes (Van Dam et al., 2002). Herbs and seeds of many plant-based foods are often evaluated to measure their levels of phenolic phytochemicals, which contain high antioxidant activity (Kwon et al., 2006). Phenolic phytochemicals are utilized by plants for their protection against abiotic and biotic stresses, but are also beneficial for human health in preventing and combating chronic diseases linked to oxidative stress (Shetty and Wahlqvist, 2004). The presence of certain phenolic compounds is linked and highly correlated with high antioxidant activity, along with high amounts of α-amylase and α-glucosidase inhibitors relevant for hyperglycemia management linked to type 2 diabetes. Therefore, natural forms of α-amylase and α-glucosidase inhibitors from plant-based foods provide dietary strategies to control postprandial hyperglycemia and could be used in therapies with minimum side effects (Kwon et al., 2006; Shetty et al., 2008; Pinto and Shetty, 2010). This has led to an increase in food-based biochemical-linked screening studies aimed at raising awareness of managing hyperglycemia and hypertension associated with type 2 diabetes through dietary means. Diets rich in fruits and vegetables also indicate a lower risk for cardiovascular complications due to their high phenolic antioxidants and natural enzyme inhibitors.

Therefore, we have developed a food-based strategy to design and evaluate novel Chilean potato (*Solanum tuberosum* ssp. *tuberosum* L.) lineage, which is widely used in the tropical and subtropical regions with a combination of select herb and spice species of Apiaceae and Lamiaceae families as condiments for their antidiabetic and antihypertensive potential. A synergistic combination of appropriate potato varieties with an appropriate combination of condiments can serve as an effective strategy for managing early stages of

type 2 diabetes, associated hyperglycemia, and its oxidation-linked micro- and macrovascular complications such as hypertension.

12.2 PHENOLIC-ENRICHED CHILEAN POTATO AND SELECT SPECIES OF APIACEAE AND LAMIACEAE FAMILIES IN DIET

Plants and especially medicinal plants and food herbs have been used as traditional medicines to treat common illnesses since ancient times. It has been estimated that presently, 80% of the world's population relies on using traditional medicine for health-care purposes (Muthu et al., 2006). Fruits and vegetables consist of nutrients such as vitamins, minerals, and dietary fiber, as well as other bioactive compounds such as polyphenols and carotenoids (Montonen et al., 2004; Robert et al., 2006), which are beneficial for human health (Shetty et al., 2008). Phytochemicals in vegetables are often found in their peels, and the content is usually higher in cultivars with brighter peel colors (Zhang et al., 2009). Phenolic phytochemicals and, specifically, secondary metabolites such as phenolics are produced by plants to provide protection for themselves to combat abiotic and biotic stresses (Shetty and Wahlqvist, 2004), but they could also prove to be beneficial for managing human chronic diseases induced by oxidative stress from high-calorie diets and/or stress-linked lifestyles. Phenolic phytochemicals are relevant to human health mainly in the form of antioxidants and associated bioactive functions for specific disease conditions (Shetty et al., 2008; Xu et al., 2009).

The potato is commonly perceived as unhealthy due to its high starch content and unhealthy style of preparation, particularly when fried in vegetable oil. But many people are not aware of its complete health benefits compared with, for example, refined cereals. Compared with common cereals such as rice and wheat, potato has a low fat and energy density, similar to legumes (Priestly, 2006; Camire et al., 2009). The peel of potatoes is considered as a good source of phytochemicals, such as anthocyanins, and its content is higher in potatoes with brighter peel colors (Zhang et al., 2009). Anthocyanins have biological functions, such as strong antioxidants, and have antimicrobial and antiobesity potential. The presence of anthocyanins are 3–4 times higher in the potato peel than in the tuber (Zhang et al., 2009). Potato is often not known as a food item with high antioxidant activity, but daily consumption can contribute to a high total phenolic content in our diet (Xu et al., 2009). Consumption of high antioxidants through fruits and vegetables could defend our bodies against cardiovascular illnesses by limiting oxidative stress (Chu et al., 2002; Robert et al., 2006).

Many important vitamins are obtained from fruits and vegetables, one of which is vitamin C. Potato is rich in vitamin C, with a content of 15 mg/100 g of steamed potato (Robert et al., 2006). The amount of vitamin C in potato can contribute 25–30% of our daily recommended dietary allowance (Robert et al., 2006). Inclusion of potato in our diet in an appropriate amount, with sufficient high bioactive variety, and with low-fat preparation, could contribute to the prevention of oxidation-linked chronic illnesses, such as type 2 diabetes and cardiovascular diseases.

The presence of α-glucosidase inhibitors in certain plant-based foods has the potential to manage hyperglycemia linked to type 2 diabetes. About 90–95% of all diabetic cases are suffering from type 2 diabetes; therefore, it is important that dietary α-glucosidase inhibitors are present in our diet to slow the absorption of glucose in the small intestine (Ranilla et al., 2010). Hyperglycemia linked to onset of type 2 diabetes could be controlled

by inhibiting α-glucosidase enzyme, which participates in overall digestion of carbohydrates. Potato varieties containing high α-glucosidase inhibitors could further be validated in *in vivo* experiments as part of a potential therapeutic strategy for managing hyperglycemia linked to type 2 diabetes. Another inhibitor related to α-glucosidase inhibitor is α-amylase inhibitor, which is known to play an important role in managing hyperglycemia associated with type 2 diabetes (Pinto et al., 2009). Inhibitory activities toward these enzymes are often associated with certain phenolic compounds present in plant-based foods. The inhibition of these key enzymes may vary due to dependence on the presence of phenolic phytochemicals that enhance their inhibitory activities in specific species and varieties (Kwon et al., 2006; Pinto et al., 2009). Pinto et al. (2009) suggested that the presence of both α-glucosidase and α-amylase inhibitory activities does not have to be present to help manage hyperglycemia associated with type 2 diabetes. Plant-based foods with mild α-amylase inhibition and strong α-glucosidase inhibition are considered ideal for avoiding digestive complications from undigested starch (Kwon et al., 2006).

Cardiovascular diseases and their complications are one of the leading causes of death worldwide, and type 2 diabetes increases the risk of cardiovascular diseases due to micro- and macrovascular complications. Inclusion of diets with fruits and vegetables helps reduce the overall risk of cardiovascular diseases due to the combination of protective micronutrients, antioxidants, phyochemicals, and fiber present in them (Liu et al., 2000). Angiotensin-converting enzyme (ACE) is known as an important target for managing vascular complications and therefore is used for treating vascular illnesses associated with cardiovascular complications. ACE regulates vascular hypertension by two different reactions. One way is to convert angiotensin I into a potent vasoconstrictor, angiotensin II, and a vasodilator bradykinin that helps lower blood pressure (Johnston, 1992; Pinto et al., 2009). High total phenolic content could inhibit ACE, which would indicate that regular intake of phenolic compounds through the diet may have beneficial effects on the cardiovascular system. Phenolic compounds such as chlorogenic acid forms 90% of the total phenolic compounds in potato tuber (Dao and Friedman, 1992) and has the ability to lower blood pressure in hypertensive patients (Cheplick et al., 2010). Other phenolic compounds such as ferulic acid, caffeic acid, and catechins are present in different varieties of potato, which has the potential to help reduce the overall risk of hypertension associated with type 2 diabetes (Li et al., 2005; Matsui et al., 2007; Nagao et al., 2007; Zhao and Moghadasian, 2008). Therefore, regular intake of certain whole potato varieties in the diet has potential health benefits by managing hyperglycemia and hypertension linked to type 2 diabetes.

In general, the use of plant-based food sources including seeds and herbs in traditional medicine has increased the interest in exploring their potential beneficial effects on human health. It has been estimated that about 70% of the world's population uses traditional medicine obtained from plants to treat and cure illnesses (Jiofack et al., 2009). Plants belonging to the Apiaceae family are often used as food, food flavorings, and for their medicinal purposes. The seeds from Apiaceae family in particular are used as a household remedy for complications such as high blood pressure (Gilani et al., 2005). In regions such as India, Apiaceae family plants are used to treat common ailments such as abdominal pain and acidity (Shekhawat and Batra, 2006). In parts of the world such as Cameroon, the use of plants from Apiaceae family are used as therapy for vomiting, appendicitis, ingestion, constipation, and to treat mosquito bites (Jiofack et al., 2009). An ancient form of traditional medicine, Ayurveda, uses the active ingredients from Apiaceae in treating various illnesses (Dhandapani et al., 2002). The reason for using Apiaceae plants in traditional medicine is due to their low toxicity as well as low occurrence of side effects compared to synthetic drugs. Several Apiaceae family plants have hypoglycemic effects, which

could possibly be due to insulin affect, by increasing pancreatic secretion of insulin from the cells of islets of Langerhans and its release from bound insulin (Prasanna, 2000; Dhandapani et al., 2002).

Utilizing natural herbal medicine for the prevention of blood pressure and type 2 diabetes is commonly practiced worldwide (Loizzo et al., 2008). It has been known that the high antioxidant activity potential is often due to certain phenolic compounds (Kiselova et al., 2006). Phenolic antioxidants are associated with therapeutic uses of medicinal plants in managing hyperglycemia, by delaying the development of type 2 diabetes. An excellent rationale for using herb-based foods for medicinal purposes is because their high bioactive function and vitamin content are correlated with high total phenolic content and total antioxidant activity. Among the many advantages of herbs as antioxidants in the diet is their slowing down of the oxidation of fats (Yen and Duh, 1994) and their free radical or active oxygen scavenger characteristics (Oktay et al., 2003). Regular intake of herbal antioxidants in our diet through various food sources could lead to weight loss and controlling obesity, which are associated with type 2 diabetes. Natural forms of herbal antioxidants have the capability to potentially provide protection against free radicals and chronic illnesses (Oktay et al., 2003), while synthetic sources of antioxidants are in limited use due to their potential carcinogenicity (Zheng and Wang, 2001). α-Glucosidase inhibition for select species of Apaiceae family suggests that they have the potential to delay the degradation of oligosaccharides, decreasing the absorption of glucose and inhibiting the increase in postprandial hyperglycemia (Loizzo et al., 2008). This α-glucosidase inhibitory activity is often associated with high total phenolic content and antioxidant activity, suggesting that certain phenolic phytochemicals are responsible for this action. The opposite has been noticed for α-amylase inhibition, where high inhibition is not associated with high total phenolic content or total antioxidant activity (Cheplick et al., 2010). Even though α-amylase inhibition has a positive effect on preventing hyperglycemia associated with type 2 diabetes, excess α-amylase inhibitors could lead to stomach distention and discomfort (Cheplick et al., 2010). Since there is a mild α-amylase and good α-glucosidase inhibition seen for certain select species of Apiaceae family, it is considered a good candidate for managing early-stage hyperglycemia associated with type 2 diabetes.

Hypertension is known as one of the risk factors for vascular complications linked to type 2 diabetes (Kwon et al., 2006) and could be managed by functional ingredients from Apiaceae and related seed and herbal extracts. Type 2 diabetes plays a role in elevating plasma lipids, serving as a risk factor for coronary heart diseases (Chaterjea and Shinde, 1994; Dhandapani et al., 2002). By applying Apiaceae seed and herbal dietary strategies, the risk of vascular complications could be reduced by lowering plasma lipid levels (Kannel and McGee, 1979; Scott, 1999; Dhandapani et al., 2002). Various phenolic phytochemicals have been known to be present in select species of family Apiaceae, which suggests its disease-prevention potential for type 2 diabetes and vascular problems. Some of the phenolic compounds observed in Apiaceae family include catechins, caffeic acid, rutin, and rosmarinic acid, which indicate that select species have the ability to provide protection against oxidation-linked diseases. Diets and medicinal formulations using seeds and leaves belonging to Apiaceae family thus can be beneficial due to its hyperglycemic inhibitory activities, associated with type 2 diabetes.

Herbs from the Lamiaceae family have been studied for their medicinal and beneficial properties associated with human health. Many plants belonging to the Lamiaceae family have originated from the Middle Eastern region in Asia, and are used as traditional medicines to treat common colds and stomach disorders (Cuvelier et al., 1996). *Origanum majorana* (marjoram), which belongs to the Lamiaceae family, is commonly used as a folk

medicine, particularly in the form of tea, which is prescribed for fever and sinus congestion, and is used as well to treat nervous disorders (Qari, 2008). Plants from the Lamiaceae family are known to be rich in essential oils, which could indicate antibacterial, antimicrobial, and suppressive activities against tumor formation (Hilan et al., 1997; Farhat et al., 2001). Herb species belonging to the Lamiaceae family also have antihepatoma and antigenotoxicity characteristics, which could reduce the number of chromosomal aberrations (Qari, 2008), and various studies have determined their importance in managing type 2 diabetes. Lamiaceae family herbs are a rich source of phenolic phytochemicals and antioxidants (Kwon et al., 2006), which are highly correlated with α-glucosidase inhibitors, playing a potential role in hyperglycemia management. Lamiaceae family plants are good sources of natural antioxidants, therefore potentially managing chronic oxidation-linked complications, such as cardiovascular diseases and type 2 diabetes (Shetty, 1997; Kwon et al., 2006). The antioxidant activity linked with phytochemicals is also linked to lowering mortality rates for cancer in humans (Velioglu et al., 1998).

Preparing samples through aqueous extracts often indicated higher phenolic content, which could be due to high temperatures of sample preparation, compared with ethanolic extracts (Seaberg et al., 2003; Chun et al., 2005). High antioxidant activity linked with total phenolic content allows the consumption of plant-based food from the Lamiaceae family in the diet to delay or prevent the oxidizing chain reaction (Zheng and Wang, 2001; Chun et al., 2005). The natural sources of dietary phenolic antioxidants from Lamiaceae are considered to be important for potentially delaying the development of chronic diseases, such as cardiovascular complications and cancers (Shetty, 1997; Akyon, 2002; Chun et al., 2005). Lamiaceae compounds may not be the only source of high antioxidant activity, as it could be the combination of redox properties, physicochemical structure, and nature of the individual phenolics (Kahkonen et al., 1999; Zheng and Wang, 2001; Parejo et al., 2002; Chun et al., 2005). The ability of plant-based additives from the Lamiaceae family to prevent oxidative stress suggests that the substitution of natural plant extracts for synthetic sources of antioxidants also has the potential to influence human health (Martinez-Tome et al., 2001; Hinneburg et al., 2006). These herbs could be industrially efficient as food antioxidants for prevention of oxidative stress due to their high phenolic antioxidants, which can slow down the oxidative degradation of lipids (Wojdylo et al., 2007), as well as improve the overall stability of certain foods in the postharvest stage. Further pancreatic beta cells could be damaged due to oxidative stress before they proliferate. If these damaged cells could be prevented from proliferating through cell repair or apoptosis, then the incidence of diabetes could be reduced. The high antioxidant activity of Lamiaceae herbs and their use as food ingredients could suppress the oxidative stress caused to pancreatic beta cells, reducing the risk of diabetes (Song et al., 2005; Bhandari et al., 2008). Lamiaceae family plants contain certain phenolic phytochemicals, such as rosmarinic acid, caffeic acid, and rutin, which may have hypoglycemic effects due to high α-glucosidase inhibitory activities. Phenolic compounds such as rosmarinic acid have high antioxidant activity, providing protection against oxidation-linked illnesses (Peterson and Simmonds, 2003). Intake of high rosmarinic acid-containing herbs of the Lamiaceae family could possibly result in enhancing health, due to their potential benefits in terms of α-glucosidase inhibition relevant to hyperglycemia associated with type 2 diabetes. Thus, consumption of food containing Lamiaceae herbs could reduce blood glucose concentration and lengthen the duration of carbohydrate absorption (Ye et al., 2002). Clinical information from the functionalities of plants belonging to this family could be further applied to *in vivo* studies for development of innovative ingredient designs and formulations, for

therapeutic strategies, and to prevent chronic illnesses associated with type 2 diabetes and its complications.

12.3 COMBINATION OF POTATO WITH SEEDS AND/OR HERBS FOR HYPERTENSION AND HYPERGLYCEMIA MANAGEMENT

In this food design study, the combination of three different plant-based foods was evaluated for their functionalities that could be targeted against diet-linked chronic illnesses such as type 2 diabetes and associated complications. First, we evaluated the phenolic-linked antidiabetic potentials of 54 subtropical varieties of Chilean potato. Second, we investigated functionalities of seeds of six select species of the Apiaceae family and how their *in vitro* effects could provide a biochemical rationale to target them toward preventing and managing hyperglycemia and hypertension associated with type 2 diabetes. Third, we screened two species belonging to the Lamiaceae family from the Near East Asia region for their antidiabetic and antihypertensive potential. A combination of these herbs in a potato whole food design could yield multiple benefits for managing early stages of type 2 diabetes and its complications.

12.3.1 Chilean potato (*Solanum tuberosum* ssp. *tubersocum* L.)

The potato originated from the Andean mountains (Andean potato, *Solanum tuberosum* ssp. *andigenum* L.) and Chiloe Islands (Chilean potato, *Solanum tubersoum* ssp. *tuberosum* L.) of South America. Initially, we evaluated the total soluble phenolic content and antioxidant activity of the 54 different varieties of Chilean potato (Saleem et al., unpublished results). The bright red, purple, and pink colors of the potato indicated higher soluble phenolic content and antioxidant activity, compared with colors such as white and yellow. Biologically relevant functions such as high antioxidant activity have been potentially due to the presence of anthocyanins. The brighter color of potatoes is considered to be an indication of anthocyanin presence, which is 3–4 times higher in potato peel compared with the tuber (Zhang et al., 2009). Therefore, we can assume that different peel colors of the potato affect the activity of antioxidants in a particular sample. Table 12.1 indicates the results for six random varieties of the 54 samples of Chilean potato we investigated. These results confirm that the color of a particular potato variety is correlated with high or low total soluble

Table 12.1. Total phenolic content (mg GAE/g DW) and total antioxidant activity (% DPPH inhibition) correlation with six Chilean potato sample colors

Sample	Color	Total phenolics (mg GAE/g DW)	% DPPH inhibition
PA 4	Red	13	69
PA 6	Red	12	74
PA 7	Red	11	75
PA 10	White-yellowish	7	58
PA 15	White	3	23
PA 43	Yellow	1	10

GAE, gallic acid equivalents.

Table 12.2. α-Glucosidase inhibition (% inhibition) and total antioxidant activity (% DPPH inhibition) correlation with six Chilean potato sample colors

Sample	Color	α-Glucosidase % inhibition	% DPPH inhibition
PA 5	Red	54	75
PA 13	Purple	59	65
PA 14	Purple	53	65
PA 29	Yellow	1	6
PA 35	Yellow	0.000	21
PA 54	Yellow	0.000	12

phenolic content and antioxidant activity. These results also indicate that the total soluble phenolic content is highly correlated ($r = 0.83$) with total antioxidant activity of all 54 Chilean potato varieties (Saleem et al., unpublished results). This high correlation suggests that high total antioxidant activity (% 2,2-diphenyl-1-picrylhydrazyl [DPPH] inhibition) could be due to the presence of certain phenolic compounds in specific varieties. Therefore, we can conclude that specific Chilean potato varieties with high antioxidant activity could be beneficial toward limiting oxidative stress and help in managing micro- and macrovascular oxidative stress-linked complications of hyperglycemia and hypertension linked to type 2 diabetes.

Early stages of type 2 diabetes could be controlled via inhibition of α-glucosidase enzyme, which is essential in overall digestion and uptake of carbohydrates from the diet. Therefore, we investigated α-glucosidase inhibition of all 54 Chilean potato varieties, so they could further be targeted for therapeutic or clinical strategy for managing hyperglycemia associated with type 2 diabetes. The results obtained for α-glucosidase inhibition indicated a moderate correlation with total soluble phenolic content ($r = 0.50$) and antioxidant activity ($r = 0.54$). Since a moderately good correlation was observed between total soluble phenolic content and antioxidant activity for α-glucosidase inhibition, we suggest that the antioxidant activity and soluble phenolic content could play a critical role in determining high or low α-glucosidase inhibition. Also, these correlations may suggest that the higher inhibition of α-glucosidase could be due to the brighter colors of certain potato varieties. Table 12.2 represents the results for six random varieties, which indicates that the brighter color of potato correlated with a higher α-glucosidase inhibition as well as high total antioxidant activity. Also, it indicated that the sample with no α-glucosidase inhibition had low antioxidant activity; these samples had lighter colors, such as yellow. Therefore, α-glucosidase inhibitors in different skin samples of Chilean potato could be targeted in therapeutic strategies to manage hyperglycemia associated with type 2 diabetes.

No α-amylase inhibitory activity was found in any of the 54 Chilean potato samples; α-amylase inhibition may be due to some other phenolic compounds or nonphenolic bioactive compounds that are not present in Chilean potato samples we studied. It has been suggested that a mild α-amylase inhibition and a good α-glucosidase inhibition is considered good for treating early-stage hyperglycemia (Pinto et al., 2009) and could be considered as part of a whole food design that can be part of our diet to help manage hyperglycemia linked to type 2 diabetes in its early stages (Shetty et al., 2008; Pinto and Shetty, 2010).

ACE inhibition plays a crucial role in managing hypertension, and in this study we have evaluated ACE inhibition in response to aqueous potato extracts. This study would also give us an insight into how important the varying colors of Chilean potato are in the context of antihypertensive characteristics of specific varieties. The results obtained for ACE inhibition indicated a low correlation ($r = 0.13$) for total phenolic content and a low correlation

($r = 0.21$) for antioxidant activity. The correlations observed for ACE inhibition suggests the possibility of the presence of other phenolic compounds or nonphenolic bioactive compounds in Chilean potato samples that are linked to antihypertensive potential. ACE inhibitory activity showed a high variation with inhibition as high as 88% for variety PA 17, but also indicated no inhibition for 19 variety samples. Five phenolic compounds were found in the potato samples that were studied: chlorogenic acid, ferulic acid, caffeic acid, *p*-coumaric acid, and catechin. Regular intake of these phenolic phytochemicals through our diet could reduce the risk of oxidation-linked chronic illnesses and have a beneficial affect on our overall health. Chlorogenic acid was observed to have the highest activity of all the phenolic compounds in all potato samples, at 19.3 mg/g of sample dry weight (DW) for PA 11. PA 11 also had high total phenolic content and antioxidant activity as well as α-glucosidase inhibition, but a low ACE inhibitory activity. Chlorogenic acid is known to comprise about 90% of total phenolic compounds of potato tubers (Dao and Friedman, 1992) and might play an important role in the quality and safety of the potato plant. Appropriate inclusion of all these phenolic compounds from different potato varieties could serve as beneficial for human health. Therefore, inclusion of the appropriate subtropical cultivars of Chilean potato could be beneficial in preventing hyperglycemia and hypertension in the early stages of type 2 diabetes and beneficial for the associated cardiovascular disease challenges in the long term.

The results of this study indicate that Chilean potato does not have α-amylase inhibitory activity, but the α-glucosidase inhibitory activity showed a large variation: the highest inhibition reached up to almost 60%. This indicates that certain potato varieties with these characteristics could play a role in preventing hyperglycemia linked to type 2 diabetes. Also, some varieties had high ACE inhibitory activity, suggesting that certain potato samples should be considered when designing breeding programs and diets to prevent hyperglycemia and hypertension linked to type 2 diabetes.

12.3.2 Apiaceae family

Apiaceae family plants have been commonly used as foods, food flavorings, and medicines. The seeds serve as a common household remedy for many complications such as hypertension (Gilani et al., 2005). In this study we investigated the functionalities of seeds of six select species of Apaiaceae family, and how their *in vitro* effects could provide a biochemical rationale to target them toward the prevention and management of hyperglycemia and hypertension associated with type 2 diabetes (Saleem et al., unpublished results). To screen the antihyperglycemic and antihypertensive potentials of Apaiceae family, we focused on both the aqueous and ethanolic extracts of samples of six targeted species. Table 12.3 shows the results for total soluble phenolic content and total antioxidant activity for both aqueous and ethanolic extracts of these species. Ajowan seed extracts had consistently high soluble phenolic content for aqueous and ethanolic extracts, whereas caraway and coriander had the lowest phenolic content (Saleem et al., unpublished results). High DPPH inhibition was seen for all the aqueous extracts with the highest inhibition being for dill, and the highest inhibition was seen for ajowan in the ethanolic extracts. A moderate correlation ($r = 0.61$) was found for the aqueous extracts of total soluble phenolic content and antioxidant activity by DPPH assay. The ethanolic extracts indicated a higher correlation ($r = 0.73$) for total soluble phenolic content and antioxidant activity, which suggests that the high amount of antioxidant activity may be due to the phenolic content in each seed extract of this family. This study suggests that species such as dill and ajowan could be used as food condiments

Table 12.3. Total phenolic content (mg GAE/g DW) and total antioxidant activity (% DPPH inhibition) correlation for aqueous and ethanolic extracts

Sample	Total phenolics (mg GAE/g DW)	% DPPH inhibition
	H_2O–Ethanol	H_2O–Ethanol
Dill	6.9–4.9	76.5–68.0
Ajowan	8.3–8.0	74.0–69.0
Fennel	6.0–3.7	73.3–58.2
Caraway	5.0–2.4	63.0–38.0
Coriander	4.8–2.5	71.0–25.0
Anise	5.6–4.0	71.2–65.0

Table 12.4. α-Glucosidase and α-amylase inhibition for aqueous and ethanolic extracts of the six select species of Apiaceae family

Sample	α-Glusoidase % inhibition	α-Amylase % inhibition
	H_2O–Ethanol	H_2O–Ethanol
Dill	50.0–31.5	24.3–15.0
Ajowan	49.5–33.0	0.00–0.00
Fennel	16.7–13.7	16.0–14.5
Caraway	16.5–20	32.3–10.0
Coriander	0.00–11.6	17.0–30.0
Anise	0.00–12.5	21.7–13.5

in our diet to manage chronic disease states or as medicinal material for replacing synthetic antioxidants. These could also be used as condiments with starch foods such as potato products to enhance bioactive phenolic profiles.

Total soluble phenolic content indicates a high correlation ($r = 0.86$) with α-glucosidase inhibition for aqueous extracts, and a high correlation ($r = 0.76$) for ethanolic extracts (Saleem et al., unpublished results). These results suggest that α-glucosidase inhibitory activity may have a high dependence on total soluble phenolic content of the particular species of the Apiaceae family. Table 12.4 indicates the percentage inhibition obtained for both α-glucosidase and α-amylase enzyme for the aqueous and ethanolic extracts. Antioxidant activity indicates a low correlation ($r = 0.48$) for the aqueous extracts and a moderate correlation ($r = 0.55$) for ethanolic extracts. The low correlation between α-glucosidase inhibition and antioxidant activity suggests that there may be other phenolic or nonphenolic bioactive compounds responsible for α-glucosidase inhibitory activity. Table 12.4 also indicates the results for α-amylase inhibitory activity, which are generally lower than α-glucosidase inhibitory activity of various species in the Apiaceae family. The total phenolic content and antioxidant activity for aqueous and ethanolic extracts indicates an inverse correlation between these bioactive functions and α-amylase inhibitory activity. This suggests that the presence of certain phenolic compounds and antioxidants does not have any influence on α-amylase inhibitory activity. A mild α-amylase inhibitory activity and a higher α-glucosidase inhibitory activity was observed, which could suggest that these select species from this family have the potential for managing early-stage hyperglycemia linked to type 2 diabetes (Saleem et al., unpublished results).

No ACE inhibition was observed for aqueous or ethanolic extracts of any of the select species of this family. But the HPLC analysis of phenolic phytochemicals indicated the

presence of eight phenolic compounds (Saleem et al., unpublished results). These compounds included caffeic acid, catechin, rutin, chlorogenic acid, gallic acid, ferulic acid, and rosmarinic acid. The highest content of phenolic compound observed was rutin in the aqueous (27.6 mg/g) and ethanolic (17.8 mg/g) seed extracts of dill. The presence of many different phenolic compounds suggests that select species of this family could provide protection against oxidation-linked diseases and the hyperglycemia and hypertension associated with type 2 diabetes.

This approach provides the *in vitro* biochemical rationale for *in vivo* studies of development of whole food therapeutic strategies to help prevent chronic hyperglycemia and associated complications linked to management of type 2 diabetes.

12.3.3 Lamiaceae family

Food herbs are considered a good source of phenolic phytochemicals, having a high antioxidant activity (Kwon et al., 2006). Plants of the Lamiaceae family are often used for food preservation and flavoring and for treating common illnesses (Shetty, 1997; Kwon et al., 2006). These herbs contain phenolic phytochemicals, which are beneficial for human health (Shetty, 1997). We screened phenolic-linked bioactive functions of marjoram (*Origanum majorana*) and sage (*Salvia libanotica*), belonging to the Lamiaceae family from the Near East Asia region, with a focus on their antidiabetic potential (Saleem et al., unpublished results). The total phenolic content observed for ethanolic extracts was lower than that observed for the aqueous extracts. This could be due to the lower temperatures used in the ethanolic extraction process. An inverse correlation ($r = -1$) was seen between total phenolic content and antioxidant activity for the aqueous extracts, suggesting that the phenolic content in this case may not be important in determining antioxidant activity. However, a high correlation ($r = -1$) was observed between total phenolic content and antioxidant activity for ethanolic extracts. Table 12.5 indicates the results for total phenolic content and antioxidant activity for both aqueous and ethanolic extracts. The high antioxidant activity suggests that phenolic phytochemicals from the Lamiaceae family could prevent oxidative stress-linked chronic disease malfunction. Findings such as this, where high antioxidant activity is observed for herb extracts, suggest that these could be used as substitutes for synthetic food antioxidants with additional health benefits.

Table 12.6 indicates the α-glucosidase inhibitory activity for both aqueous and ethanolic extracts of marjoram and sage, as well as the total soluble phenolic content and antioxidant activity (Saleem et al., unpublished results). Total phenolic content for aqueous extracts indicated a high correlation ($r = 1$) with α-glucosidase inhibition, suggesting the presence of certain phenolic compounds being responsible for its high inhibitory activity. The presence of rosmarinic acid indicated a high activity for the aqueous extracts reflected in a

Table 12.5. Total phenolic content (mg GAE/g DW) and total antioxidant activity (% DPPH inhibition) of aqueous and ethanolic extracts

Sample	Total phenolics (mg GAE/g DW)	% DPPH inhibition
	H_2O–Ethanol	H_2O–Ethanol
Origanum majorana	41.2–22.2	77.5–79.6
Salvia libanotica	38.4–16.8	79.0–76.6

Table 12.6. α-Glusoidase inhibitory activity and its correlation with total phenolic content and antioxidant activity for aqueous and ethanolic extracts

Sample	α-Glusoidase % inhibition	Total phenolics (mg GAE/g DW)	% DPPH inhibition
	H_2O–Ethanol	H_2O–Ethanol	H2O–Ethanol
Origanum majorana	85.6–69.3	41.2–22.2	77.5–79.6
Salvia libanotica	76.7–79.0	38.4–16.8	79.0–76.6

higher α-glucosidase inhibitory activity. However, an inverse correlation ($r = -1$) was seen for ethanolic extracts, and the amount of rosmarinic acid was significantly lower for ethanolic extracts compared with aqueous extracts. This could possibly suggest that the Lamiaceae family with phenolic compounds such as rosmarinic acid may have hypoglycemic affects due to the high α-glucosidase inhibitory activities observed. An inverse correlation ($r = -1$) was seen between antioxidant activity and α-glucosidase inhibition for both the aqueous and ethanolic extracts. This suggests that even though the percentage DPPH inhibition was seen to have high activity for both aqueous and ethanolic extracts, the antioxidant activity may not affect the α-glucosidase inhibitory activity (Saleem et al., unpublished results). These results suggest that enrichment of Lamiaceae herbs through the diet in the form of condiments or other ways could result in enhancing our health through functional phenolic bioactive enrichment of foods. Inclusion of these herbs in our diet could reduce blood glucose levels and lengthen the duration of carbohydrate absorption due to high α-glucosidase inhibitory activity.

No α-amylase inhibition was observed for ethanolic or aqueous extracts of marjoram and sage. This could be due to the absence of certain phenolic phytochemicals, causing the modulation of α-amylase inhibition. Though no α-amylase inhibition was observed, high α-glucosidase inhibition suggests that it could still serve as a food ingredient and condiment that could be targeted against treating hyperglycemia linked to type 2 diabetes. No ACE inhibition was observed, suggesting that there may be other phenolic compounds or peptides not enriched in Lamiaceae plant extracts associated with ACE inhibition (Kwon et al., 2006).

High-performance liquid chromatography (HPLC) analysis of phenolic phytochemicals showed the presence of three major phenolic compounds, which included rosmarinic acid, caffeic acid, and rutin, in both aqueous and ethanolic extracts. The content of rosmarinic acid in aqueous extracts was higher than in ethanolic extracts, thus a higher α-glucosidase inhibitory activity was seen for the aqueous extracts compared with ethanolic extracts. Additional *in vivo* studies would allow us to understand the antidiabetic and chemopreventive affects of these phenolic compounds on human health.

12.4 CONCLUSIONS: COMBINING THE CHILEAN POTATO WITH SEEDS AND HERBS FROM THE APIACEAE AND LAMIACEAE FAMILIES

The combination of an appropriate Chilean potato variety with appropriate seed condiments from the Apiaceae family and herbs from the Lamiaceae family could provide a healthy

food for management of hyperglycemia and hypertension associated with type 2 diabetes. We will make specific cultivar recommendations as well as give us insights on using natural sources of seeds and herbs for culinary purposes that could benefit human health.

For example, we can use Chilean potato varieties PA 5 and PA 11 due to their high content of antioxidants and other digestive enzyme inhibitors (Saleem et al., unpublished results). Variety PA 5 has a soluble phenolic content of almost 13 mg/g DW and high antioxidant activity with a DPPH inhibition of 75%. Although no α-amylase inhibitory activity was observed for any of the 54 varieties of Chilean potato, a high α-glucosidase inhibition was observed with 53.8% inhibition for PA 5. ACE inhibition varied between all the potato samples and ranged up to 63% inhibition for PA 5. HPLC analysis of phenolic phytochemicals indicated all five phenolic compounds that were observed for for PA 5. Chlorogenic acid, which is known to have a high activity in the potato tuber, showed the highest content of all the phenolic compounds in PA 5, with a content of almost 16 mg/g DW. PA 11 was observed to have moderate soluble phenolic content with 7.3 mg/g of DW and a high antioxidant activity of 68% DPPH inhibition. A high α-glucosidase inhibitory activity of almost 52% was observed for PA 11, as well as a high ACE inhibitory activity of 66% inhibition. HPLC analysis of phenolic phytochemicals showed PA 11 to have the highest content of chlorogenic acid with 19.3 mg/g DW, and a moderate content for other phenolic compounds as well.

A combination of PA 5 and PA 11 with aqueous seed extracts of dill and ajowan could be beneficial for human health as a substitute for other spices and especially with the use of reduced salt. The combination of aqueous seed extracts of the Apiaceae family showed significantly higher antidiabetic functionalities compared with ethanolic extracts. Dill seed extracts showed a soluble phenolic content of 6.8 mg/g DW and a high antioxidant activity with 76.5% inhibition. A moderate α-amylase inhibitory activity of 24.3% inhibition with a good α-glucosidase inhibitory activity of 49.8% inhibition was observed in dill, while no ACE inhibition was found. HPLC analysis of aqueous dill seed extracts indicated the presence of compounds such as rutin and rosmarinic acid, which are known to have hypoglycemic effects. Aqueous ajowan seed extracts indicated higher soluble phenolic content compared with dill, with a content of 8.3 mg/g DW and antioxidant activity of 74% inhibition. Although no ACE inhibition was found for any of the Apiaceae family seed extracts and no α-amylase inhibition was observed for ajowan, the α-glucosidase inhibition was observed to be high with the activity of 49.5% inhibition. HPLC analysis of ajowan showed a content similar to dill where phenolic compounds such as rutin, rosmarinic acid, caffeic acid, and catechin were observed.

Therefore, a combination of PA 5 and PA 11 with addition of dill and ajowan could lead to beneficial effects on human health. To increase the health benefits obtained from these combinations, the addition of either marjoram or sage as natural food condiments could be important as well. The aqueous herbal extracts on average showed higher antidiabetic potential for marjoram and sage compared with the ethanolic extracts. Marjoram had a higher content of total soluble phenolic with levels of 41.2 mg/g DW and high total antioxidant activity with 77.5% DPPH inhibition. Although no α-amylase and ACE inhibition were observed for marjoram, α-glucosidase inhibition showed a high activity of 85.6%. HPLC analysis of phenolic phytochemicals of the aqueous extracts of marjoram and sage showed compounds such as rosmarinic acid and caffeic acid, with rosmarinic acid having a high activity of 27.4 mg/g DW. The aqueous extracts of sage showed 39.7 mg/g DW as well as levels of caffeic acid (Saleem et al., unpublished results).

The dietary combination of appropriate potato varieties with different health benefits and appropriate varieties of seeds from the Apiaceae and Lamiaceae families could yield

health benefits against certain chronic diseases such as type 2 diabetes and associated cardiovascular complications. The use of seeds and herbs from the Apiaceae and Lamiaceae families could also replace certain high salt products that are used for culinary purposes.

Natural sources of antioxidants and certain hypoglycemic compounds can serve as an important strategy to control high blood glucose levels and the hyperglycemia associated with type 2 diabetes. Consumption of hypoglycemic compounds with the addition of ACE inhibitors in our diet and certain phenolic compounds could help in the management of micro- and macrovascular complications, and therefore provide a way of protecting against the incidence of cardiovascular diseases associated with type 2 diabetes.

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13 Fermentation-Based Processing of Food Botanicals for Mobilization of Phenolic Phytochemicals for Type 2 Diabetes Management

Chandrakant Ankolekar and Kalidas Shetty

13.1 INTRODUCTION

Fermentation can be broadly defined as a slow decomposition process by which complex organic compounds are converted into simpler compounds in the absence of oxygen. It is one of the oldest techniques by which humans have preserved food. The fermentation process produces a range of metabolic products, including organic acids, ethanol, carbon dioxide, acetic and formic acid, peptides, polysaccharides, hydrogen pexoxide, and bacteriocins. These compounds may act in combination with the fermenting microflora to produce antagonistic effects to suppress the growth of pathogenic microorganisms causing spoilage, thereby prolonging the shelf life of the food (El-Gazzar et al., 1992; Rodgers, 2001; Ross et al., 2002). Not only does fermentation increase the shelf life and decrease the need for use of other techniques of preservation, such as refrigeration, but it is also a highly cost-effective and efficient process, requiring low energy (FAO, "Fruits and vegetables: a global perspective"; Steinkraus, 1992).

Fermentation is broadly categorized into two types: submerged fermentation and solid state fermentation. Large-scale production of fermented products such as alcohol, organic acids, antibiotics, and other metabolites is achieved through submerged fermentation, whereas solid state fermentation is mostly limited to production of metabolites from fungi (Eugene and Karanth, 2006). However, aerobic growth of filamentous fungi is technically not fermentation as it involves oxidative phosphorylation and the necessity of oxygen for energy generation. Therefore, the term "fermentation" should be used only in reference to conversion of complex organic compounds where oxygen is not used for energy generation and largely restricted to bacteria and yeast but not aerobic filamentous fungi that need oxygen for cellular growth.

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13.2 DIABETES: THE RISING BURDEN

Non-insulin-dependent diabetes mellitus, which is prevalent in the developed Western world, is rapidly growing in developing countries and is reaching pandemic proportions globally. Prevalence of type 2 diabetes has almost doubled in a short span of 10 years from 1995 to 2004 (van Dieren et al., 2010). The International Diabetes Federation projects the prevalence of type 2 diabetes to rise from the current estimated 285 million in 2010 to 438 million in 2030 (International Diabetes Federation, "Diabetes"). Currently, the estimates for the number of people with diabetes living in urban areas are around 113 million; in rural areas it is estimated to be at 78 million. Although in 2030 the projected numbers for rural areas are expected to increase by less than a quarter, the number for urban areas is expected to double (International Diabetes Federation, "Diabetes"). In 2002, India was the country with highest relative number of diabetics; however, recently, China has overtaken India with 15.5% of the population being diabetic (Pradeepa and Mohan, 2002; Yang et al., 2010).

Obesity has been implicated as one of the risk factors for several noncommunicable chronic diseases such as type 2 diabetes and cardiovascular diseases such as heart disease, hypertension, and stroke (Kumanyika et al., 2002). Rapid changes in dietary habits led by socioeconomic changes in urban areas of developing countries such as India, China, and Latin America have been linked to an increase in the prevalence of type 2 diabetes in these regions (Aschner, 2002; Pradeepa and Mohan, 2002). Urbanization has led to high-calorie diets with concurrent decrease in whole cereal and fiber intake (Popkin, 2003). The availability of technology has reduced physical activity and promoted a sedentary lifestyle, which has complicated the problem. Walker et al. (2010) reported that lifestyle changes through diet and exercise have the potential to reduce type 2 diabetes by 28–59%.

Diabetes is not only dangerous because of its health implications but also because of the economic burden it places on developing societies still battling infectious diseases. The current global health-care expenditure to treat and prevent diabetes and its complications is around $376 billion, and it is projected to be close to $0.5 trillion by 2030 (International Diabetes Federation, "Diabetes").

13.3 FERMENTATION AND HEALTH: A HISTORICAL PERSPECTIVE

Archaeological evidence points to the art of fermentation first originating in Henan province in China, as early as 7000 B.C. (McGovern et al., 2004), and the earliest large scale wine production dating to 5400 B.C. in modern-day Iran (McGovern et al., 1996). From Mesopotamia fermentation technology spread to the rest of the world. The therapeutic properties of *dahi* (the Indian equivalent of yogurt) and *chhash* (stirred diluted yogurt) and their role in preventing stomach and intestinal disorders are mentioned in the Ayurveda, which is said to have been compiled around 1500 B.C. (Prajapati and Nair, 2003). Yogurt and other fermented milks were prescribed by physicians in the ancient Middle East for intestinal and stomach disorders and stimulation of appetite (Rasic and Kurmann, 1978). King Francis I of France was cured of a debilitating illness after eating yogurt made from goat's milk, and it is said that longevity and fecundity of Abraham was attributed to the regular consumption of fermented milk products such as yogurt (Deeth and Tamime, 1981).

It is reported that cheese was first developed near the Tigris and the Euphrates rivers near Iraq. Cheese has the advantage of a longer shelf life and is a more nutritious and high-energy food than milk or yogurt (Ross et al., 2002; Heller et al., 2003).

Sauerkraut, which developed using lactic acid bacterial (LAB) fermentation, has a long history of use as a food for therapeutic purposes**.** Sauerkraut is an excellent source of vitamin C and can prevent scurvy; *Lactobacillus plantarum*, the dominating bacteria, can mitigate intestinal disorders. These observations were made by British explorer James Cook, which led to a high survival rate for men on board his ships (Lloyd, 1949). Metchnikoff (1908) was the first to provide a scientific basis for the therapeutic properties of fermented food products by understanding the changes in the microecology of the gut following consumption of fermented milk. He hypothesized that the large bowel harbored microorganisms that produced toxic compounds that when absorbed by the body contributed to the process of aging. A simple solution was to modify the microflora of the large intestine by consuming fermented milk containing lactic acid bacteria that inhibit putrefactive organisms and hence decrease the toxic metabolites such as amines produced by putrefactive organisms in the bowel. He further hypothesized that the unusually long and healthy lives of Bulgarian peasants was due to the large amounts of fermented milk consumed by these people (Metchnikoff, 1903, 1908; Tannock, 1997).

Reports on the health benefits of fermented foods ranging from its antimutagenic potential to its hypocholesteromic effect has led to increased interest in research and innovations in fermented food products in recent times. Although fermentation as a technique has been used for thousands of years to preserve food and promote good health, it was not until Antonie van Leeuwenhoek invented the microscope and Pasteur developed pasteurization that fermentation as a process was attributed to the activity of microorganisms growing in the food. Although fermentation can be considered an art, and knowledge of traditional fermentation techniques was propagated orally from one generation to the next, modern large-scale productions rely on better understanding of the bacterial systems and process control for a consistent quality of the final product.

13.4 FERMENTATION: ADDING VALUE

Although fermentation has a number of functional aspects, it primarily evolved as a process to preserve food safely. Fermentation may increase the shelf life of food by one to two orders of magnitude while adding value by enriching it with nutrients, flavor, and in some cases, detoxifying the substrate. Steinkraus (1994) listed some of the major roles of fermentation.

13.4.1 Preservation of food through acid/alcohol formation

Glucose utilization takes place via two major pathways in lactic acid bacteria depending on whether the bacterium is homofermentative (only one end product, lactate) or heterofermentative (more than one end product, at least 50% lactate) (Kandler, 1983). In homofermentative bacteria, 1 mole of glucose is converted to 2 moles of pyruvate through glycolysis. This generates two ATP molecules while using up one NAD^+. NAD^+ is regenerated by reduction of an internal substrate such as pyruvate, which is converted to lactic acid. Heterofermentation leads to formation of gluconate-6-phosphate, which on decarboxylation results in the formation of xylulose-5-phosphate and carbon dioxide.

Xylulose-5-phosphate on chain splitting yields C-2 and C-3 moieties: lactate and acetate or ethanol (Kandler, 1983). Lactose is taken up by a specific permease and enters the cytoplasm where it is hydrolyzed by β-galactosidase to glucose and galactose (Thompson, 1979; Kandler, 1983). The resulting glucose along with galactose, which is converted to glucose-6-phosphate, are fermented via glycolysis. Yeast produces pyruvate through the glycolytic pathway; however, pyruvate is decarboxylated to produce acetaldehyde, which is reduced by alcohol dehydrogenase to ethanol**.** Lactic acid produced during fermentation helps preserve food by lowering the pH, whereas ethanol acts by being toxic to pathogenic microorganisms and hence severely restricts their growth in foods, keeping the food safe for long periods of time.

13.4.2 Enrichment of food substrates through formation of micro and macro nutrients

Lactic acid bacteria have to rely on their complex proteolytic systems to obtain amino acids from their substrates for synthesizing proteins and nitrogen required for growth, due to their limited amino acid synthesizing ability (Law and Kolstad, 1983; Thomas and Pritchard, 1987; Chopin, 1993). Milk serves as a good example since it has very little free amino acid (Law and Kolstad, 1983). When growing in milk, proteins are degraded to oligopeptides by an extracellular proteinase (Kunji et al., 1998). Peptidases are intracellularly located, which means peptides have to be internalized by a peptide transport system before intracellular hydrolysis of these peptides to amino acids can take place (Juillard et al., 1998). These amino acids can be used for protein synthesis and other metabolic activities (Mierau et al., 1997). Both extracellular proteinases and intracellular peptidases work in combination to produce peptides and free amino acids, which contribute to the texture and flavor of fermented milk products (Steele and Ünlü, 1992; Mierau et al., 1997; Mcsweeney and Sousa, 2000).

Changes in vitamin content depend on the substrate, microorganism involved, length of fermentation, and fermentation conditions. In most cases there is an improvement in the content of B-group vitamins. During cereal and cereal-legume blend fermentation there was an increase in thiamin, riboflavin, and niacin content by 1- to 2.5-fold in all cases except in rice fermentation where thiamin and niacin were found to decrease (Tongnual and Fields, 1979; Aliya and Geervani, 1981; Kazanas and Fields, 1981). During soybean fermentation (combination of aerobic growth and fermentation using mixed cultures) to tempeh, the riboflavin and nicotinic acid content were seen to increase, whereas the thiamine content decreased to an undetectable level (Van der Riet et al., 1987).

13.4.3 Flavor, aroma, and texture development

Microorganisms growing in food cause a number of changes resulting in formation of new compounds, which are perceived as flavor components. Major contributing factors are the lipolytic and proteolytic activity of microorganisms along with metabolic products formed that add to the flavor of fermented foods. In cured hams, the secondary metabolism of flavor-improving microorganisms, which is associated with amino acid catabolism, produces aldehydes, methyl ketones, secondary alcohols, ethyl esters, all of which contribute to the flavor (Hinrichsen and Pedersen, 1995). In sauerkraut, volatile sulfur compounds such as hydrogen sulfide and dimethyl sulfide, along with carbonyls, contribute to the flavor (Lee et al., 1974). In sourdough fermentation, homofermentative bacteria mainly produce

diacetyl and other carbonyls; yeast mainly produce iso-alcohols; and heterofermentative bacteria produce ethylacetate with some alcohol and aldehydes (Damiani et al., 1996). In milk, proteolysis of milk proteins produces the taste and aroma of fermented milk (Mierau et al., 1997; Juillard et al., 1998; Kunji et al., 1998). Some strains of lactic acid bacteria can metabolize citrate, producing various aroma compounds such as diacetyl, acetaldehyde, and acetoin, which can have a significant effect on the flavor of fermented food (De Figueroa et al., 1998). Changes in texture resulting from fermentation are mostly due to proteins undergoing changes during fermentation. In milk, the lactic acid produced denatures the protein to form a three-dimensional network forming a gel. In breads, the lactic acid produced can delay retrogradation, which depends on the level of acidification and the lactic acid bacteria strain (Corsetti et al., 2000).

13.4.4 Detoxification of substrates during fermentation

Cassava is a starchy tuberous root that may contain cyanogenic glucosides, which are toxic when consumed. It is reported that cassava can be completely detoxified by the fermentation process (Olasupo, 2006). Microorganisms reportedly cause cellular breakdown and activate endogenous enzymes, which cause detoxification (Olasupo, 2006). Microorganisms can produce phytases, which can hydrolyze phytates that cause reduction in the bioavailability of iron and other minerals (Kheterpaul and Chauhan, 1991).

13.5 PHENOLIC ANTIOXIDANTS AND DIABETES MANAGEMENT

Type 2 diabetes and associated complications are reaching pandemic status and continue to increase worldwide at alarming rates. Mitochondria are the biggest source of oxidants such as superoxide, hydrogen peroxide, and hydroxyl radicals, which are continuously produced as a result of aerobic respiration (Shigenaga et al., 1994). Every cell has numerous antioxidant defenses, such as catalse, peroxidase, and superoxide dismutase, which can protect the cell against oxidants; however, oxidants that escape can cause damage to macromolecules and especially to nuclei acids, leading to mutation and cancer (Ames et al., 1993). Obesity associated with current life style changes increases oxidative stress, which has been stated to be an important pathogenic mechanism in obesity-related metabolic syndromes, one of which is type 2 diabetes (Urakawa et al., 2003; Furukawa et al., 2004). Recently, it has been reported that reactive oxygen species have a causal role in multiple forms of insulin resistance, and antioxidant therapy may be a useful strategy to control type 2 diabetes (Houstis et al., 2006). Fruits, vegetables, herbs, and other foods rich in phenolic phytochemicals with potential antioxidant activity can counteract and alleviate damage resulting from oxidative stress and insulin resistance and reduce the risk of type 2 diabetes (Dembinska-Kiec et al., 2008; Bisbal et al., 2010). Phenolic structures found in foods can stabilize reactive oxygen species by donating electrons and by delocalization of unpaired electrons on the resulting phenolic-radical derivative (Rice-Evans et al., 1997). Bisbal et al. (2010) have reviewed a list of antioxidant compounds from common food sources that exhibit beneficial activities for glucose metabolism and type 2 diabetes.

Type 2 diabetes is associated with a postprandial increase in blood glucose level, a condition known as hyperglycemia, when intestinal, pancreatic, and salivary enzymes

(α-glucosidase and α-amylase) rapidly break down soluble carbohydrates. Inhibition of intestinal α-glucosidase is a good strategy to control the postprandial rise in blood glucose since it delays the breakdown of starch and sucrose (Bischoff, 1994). There are several commercial drugs that have a similar mode of action; however, these drugs may have side effects including abdominal distention, flatulence, meteorism, and possibility diarrhea (Puls et al., 1977; Bischoff, 1994). Many phenolic compounds, such as catechin, caffeic acid, resveratrol, quercetin, protocatecheuic, anthocyanins, and rosmarinic acid, found in common foods, have been reported to have moderate to high α-glucosidase and α-amylase activity *in vitro* (Matsui et al., 2001; McCue and Shetty, 2004; Kwon et al., 2006a). Hanhineva et al. (2010) provide an excellent compilation of effect of pure phenolics and dietary plants or extracts on carbohydrate homeostasis measured *in vitro*. Phenolic phytochemicals can bring about inhibition of enzymes by binding to the active site or by blockage at several subsite of the enzymes (Randhir and Shetty, 2007). Polyphenols can bring about enzyme inhibition by formation of a polyphenol–enzyme complex at the active center through the interactions of hydroxyl groups in phenols with polar groups (amide, amino, and carboxyl group) in the enzyme (He et al., 2007).

13.6 MICROBIAL AEROBIC GROWTH AND FERMENTATION AND ITS ANTI-DIABETES POTENTIAL BY PHENOLIC AND ANTIOXIDANT MOBILIZATION

Consumption of food rich in phenolic phytochemicals with their antioxidant potential and abililty for inhibition of carbohydrate cleaving and absorbing enzymes is a good strategy for management of type 2 diabetes.

13.6.1 Solid State Growth (SSG)

SSG is typically a first-stage oxygen-dependent growth of microorganisms on organic substrates with low water content in a multistep process that has been used for several centuries for making products such as miso, natto, tempeh, and soy sauce. Recently, traditional SSG has been adopted for various dietary and nondietary substrates not only for enrichment with phytochemicals but also for adding value to agricultural and food processing waste. SSG of legumes and cereals to mobilize phenolics and antioxidants has been well documented (McCue and Shetty, 2003; McCue et al., 2004; Fernandez-Orozco et al., 2007; Pyo and Lee, 2007; Randhir and Shetty, 2007; Lee et al., 2007, 2008; Bhanja et al., 2008, 2009). Phenolic antioxidant mobilization has been attributed to the role of carbohydrate-cleaving enzymes such as β-glucosidase, α-amylase, α-glucosidase, and β-glucuronidase during SSG using food-grade aerobic fungi (McCue and Shetty, 2003; McCue et al., 2004; Randhir and Shetty, 2007; Bhanja et al., 2009). McCue and Shetty (2003), in their study on SSG of soybean with *Rhizopus oligosporus*, have noted an increase in both α- and β-glucosidase activities, which precedes the increase in total phenolic content, suggesting a possible role of these enzymes in phenolic mobilization in the early stages of aerobic growth. α-Amylase activity on the other hand was high initially and then decreased as growth proceeded, indicating a possible role in initial increase in phenolics observed during aerobic growth. β-Glucuronidase was suggested to be involved in the biotransformation of insoluble phenolics into more water-soluble forms (Hammer et al.,

2001; McCue and Shetty, 2003). Phenolic antioxidant mobilization in SSG of soybean powder with *Lentinus edodes* indicated the role of β-glucosidase, α-amylase, and laccase, a lignin-degrading enzyme, but not α-glucosidase (McCue et al., 2004). It was further reported that β-glucosidase may work in concert with laccase to increase the access to starch and other carbohydrates that are encapsulated by polymeric phenolic layers (McCue et al., 2003). The role of β-glucosidase in phenolic mobilization has been reported during SSG of mung bean and fava bean (Randhir et al., 2004; Randhir and Shetty, 2007). Recently, it was reported that enrichment of phenolics and antioxidant ability in SSG of wheat was due to the enzymatic action of β-glucosidase, α-amylase, and xylanase in the case of *Aspergillus oryzae* and xylanase and β-glucosidase in the case of *Aspergillus awamori nakazawa*, whereas in the case of rice, it was due to β-glucosidase and α-amylase (Bhanja et al., 2008, 2009). Douchi is a traditional Chinese microbially processed soybean product that has come to attention due to its potent α-glucosidase activity. Clinical studies in diabetic patients and mice studies have shown douchi to have antihyperglycemia potential through α-glucosidase inhibition (Fujita and Yamagami, 2001; Fujita et al., 2003). During growth, α-glucosidase inhibitory activity was seen to increase, which correlated well with the formation of soybean isoflavones from the glucosides of soybean, one of which—geneistin—has been identified to be a potent α-glucosidase inhibitor (Lee and Lee, 2001; Chen et al., 2007). Isoflavonoid aglycones formed during growth have greater activity than their corresponding glycosides, which may be the reason for microbially processed products being more effective at controlling glucose metabolism (Kwon et al., 2010). Chungkookjang, a fermented soybean product similar to Japanese natto, improved glucose homeostasis as compared with cooked soybean by potentiating insulin signaling via induction of IRS2 in diabetic rats (Kwon et al., 2010). Fungal-enriched cheeses have shown higher total phenolic, antioxidant, and α-amylase activity *in vitro* as compared with plain cheese, which has been attributed to the production of phenolics and specific metabolites by *Penicillium* species (Apostolidis et al., 2007b). The SSG concept applied to cranberry fruit extracts and olive pomace by-products has shown enhanced phenolic antioxidant activity, which has been linked to high β-glucosidase activity (Zheng and Shetty, 2000; Vattem and Shetty, 2002, 2003). This strategy has produced extracts with enhanced phenolic compounds that have been targeted for antimicrobial action against pathogens (Vattem et al., 2004a,b, 2005). This concept can be further extended to other dietary phenolic sources for capturing the phytochemical potential targeted at type 2 diabetes management.

13.6.2 Liquid state (submerged) fermentation

Fermentation of milk for diabetes management has been reported by various authors (Teruya et al., 2002; Apostolidis et al., 2006, 2007a; Yadav et al., 2006). The increase in phenolic content and antioxidant and α-glucosidase activity observed during milk fermentation was attributed to formation of simple phenolics (Apostolidis et al., 2007a). High levels of glucosidase correlating to total phenolics and moderate amylase activity has been reported in yogurt (Apostolidis et al., 2006). A heat- and pH-stable small molecule was revealed to be the active agent in a water-soluble fraction of kefir that increased glucose uptake in L6 myotubes both with and without insulin stimulation (Teruya et al., 2002). Similarly, supplementing a high-fructose diet of rats with dahi (the Indian analog of yogurt) resulted in the delay of glucose intolerance until the fourth and fifth week as compared with the control group, where it began in the third week (Yadav et al., 2006). Antidiabetes

functionality of soymilk fermented with kefir culture has been recently documented by Kwon et al. (2006b), in a mixed substrate that also included botanical extracts of *Rhodiola crenulata*. An increase in α-glucosidase activity has been attributed to an increase in tyrosol from *Rhodiola* supplementation. They further reported that the increase in tyrosol content was due to the action of β-glucosidase on salidroside. McCue and Shetty (2005) found a strong correlation between laccase and total peroxidase activity during phenolic antioxidant mobilization in yogurt production from soymilk using kefir cultures. An increase in antioxidant activity during soymilk fermentation using lactic acid bacteria has been attributed to hydrolysis of glycosidic isoflavones to isoflavone aglycones by β-glucosidase (Pyo et al., 2005; Chien et al., 2006; Wang et al., 2006). Papaya extracts fermented with yeast have been shown to be a potent antioxidant both *in vitro* and *in vivo* (Imao et al., 1998; Amer et al., 2008). Martin and Matar (2005) reported a significant increase in total phenolic and antioxidant capacity of lowbush blueberry juice after fermentation with *Serratia vaccinii*. The increase in phenolic compounds was thought to be due to deglycosylation of phenolic compounds and biosynthesis of new phenolics by the bacterium. Vuong et al. (2007) reported an increase in glucose uptake, with or without insulin, in myotubes and adipocytes by a lowbush blueberry juice fermented using *S. vaccinii* bacteria, whereas nonfermented juice had no effect on transport. This biotransformed juice further showed an antiobesity and antidiabetic effect in mice. This was hypothesized to be due to a significant increase in antioxidant and phenolic compounds during fermentation, which reduces oxidative stress in diabetic mice (Vuong et al., 2009). Further, this juice protected neurons against hydrogen peroxide-induced cell death, and thereby was a potent therapeutic agent against neurodegenerative diseases (Vuong et al., 2010). Berry juices when fermented with *S. vaccinii* showed increased antioxidant activity and inhibited LPS/IFN-gamma-activated macrophage $NO^{\cdot}$ production; however, there was an increase in TNF-α production (Vuong et al., 2006).

13.7 FRUIT JUICE FERMENTATION FOR HEALTHY FOOD INGREDIENTS FOR MANAGEMENT OF TYPE 2 DIABETES

Fermentation of milk and soymilk with lactic acid bacteria and kefir cultures have shown good potential for type 2 diabetes management (Kwon et al., 2006b; Apostolidis et al., 2007a). We extended this fermentation concept to the *Rosaceae* family fruit juices to check their antihyperglycemia and antihypertensive potentials. Fresh pressed juice (100%) was adjusted to a pH of 6 and then fermented with *Lactobacillus acidophilus* for 72 hours, and changes in total soluble phenolic, antioxidant potential along with inhibition of key starch breakdown and uptake enzymes were determined every 24 hours. All the assays were carried out by adjusting the pH and at fermented acidic pH. In the case of cherry, fermentation was also carried out without initial adjustment of the pH.

13.7.1 Apple juice fermentation

During apple juice fermentation with lactic acid bacteria, a decrease in total phenolics was observed after 72 hours. The total amount of phenolics present in the fermenting substrate is a result of phenolic mobilization linked to a flux between the formation/degradation of polymeric phenolics and the liberation of free phenolics (McCue and Shetty, 2005). The

decrease could also be due to degradation of phenolic compounds as a possible detoxification mechanism for lactic acid bacteria or due to formation of large polymeric phenolics from simple phenolics during fermentation. The antioxidant value decreased overall for all samples after 72 hours, with a temporary spike at 48 hours for most samples. The decrease in antioxidant activity may be attributed to the degradation of phenolic structures during fermentation. The temporary increase may be due to hydrolysis of glycosidic bonds, resulting in an increase of aglycone structures, which have higher antioxidant activity (Pyo et al., 2005; Chien et al., 2006; Wang et al., 2006). The increase in antioxidant activity might also be due to formation of polymeric phenolics (McCue and Shetty, 2005). α-Glucosidase inhibition decreased overall for pH-adjusted samples, whereas it increased for fermented acidic samples. α -Glucosidase inhibitory activity in the fermented samples was hypothesized to be a result of the combination of phenolics and pH. In the pH-adjusted samples, the decrease in α-glucosidase inhibitory activity could be because of degradation of simple phenolics (Apostolidis et al., 2006; Kwon et al., 2006b) or because the compounds formed due to polymerization of simple phenolics may not be equally effective in inhibiting α-glucosidase. The increase in the 48 hours fermented sample may be because of formation of free phenolic due to degradation of large polymeric phenolics (Apostolidis et al., 2007a). In most cases, fermentation maintained constant α-amylase inhibition or caused an overall slight decrease in inhibition. Amylase inhibition was attributed only to changes in phenolics, since lactic acid has been shown to have no effect on amylase inhibition (Apostolidis et al., 2007a).

13.7.2 Pear juice fermentation

A decrease in total phenolics was observed after 72 hours of fermentation. Similar causes of phenolic flux such as formation/degradation, degradation by bacteria, or formation of polymeric phenolics can be attributed to the decrease. Antioxidant activity decreased or remained constant for all fermented samples after 72 hours, with a temporary spike at 48 hours. The decrease may be due to degradation of phenolics, whereas the increase at 48 hours could be due to aglycone formation as well as formation of polymeric phenolics. α-Glucosidase inhibition remained constant or increased for pH-adjusted samples, whereas it increased significantly for pH not adjusted (fermented acidic) samples. No α-amylase inhibition was found in fresh or fermented pear juice.

13.7.3 Cherry juice fermentation

The total phenolics decreased as in apple and pear juice fermentation with similar pH treatment. However, when the initial pH was not adjusted and the final pH was adjusted, total phenolics increased in the sample. This was mostly an effect of the pH. When the initial and final pH were not adjusted, the total phenolics remained constant. Friedman and Jurgens (2000) suggested that a pH >7 could cause changes in the spectrum of phenolic compounds. Multiring aromatic structures such as catechin, epicatechin, and rutin are more resistant to such changes. Although the pH range tested in their work was high, these results might explain the difference in total phenolics assayed at fermented acidic pH and when the final pH is adjusted. The antioxidant activity was a function of the final pH of the sample. In cases where the final pH was adjusted, the antioxidant activity decreased, whereas when analysis was carried out at fermented acidic pH, antioxidant activity increased. This could be due to the changes in phenolic structures caused by adjusting the pH. α-Glucosidase

activity increased only when the final and initial pH were not adjusted; for all other samples it decreased or remained constant. α-Amylase inhibition was not found in fresh or fermented cherry juice.

13.8 SUMMARY

Overall it is clear that fermentation of fruit juice substrates or botanicals such as soymilk can enhance phenolic-linked bioactive compounds that have a potential for management of hyperglycemia of early-stage type 2 diabetes and other complications such as hypertension. Therefore, fermented juices not only can be used as bioactive ingredients for managing stages of metabolic syndrome disease but can also function as probiotics for better gut health and innate immunity. In many cases, such as in kefir fermentation, mixed cultures, including yeasts, are involved. Figure 13.1 summarizes the range of food sources, fermented products, and their health-beneficial qualities.

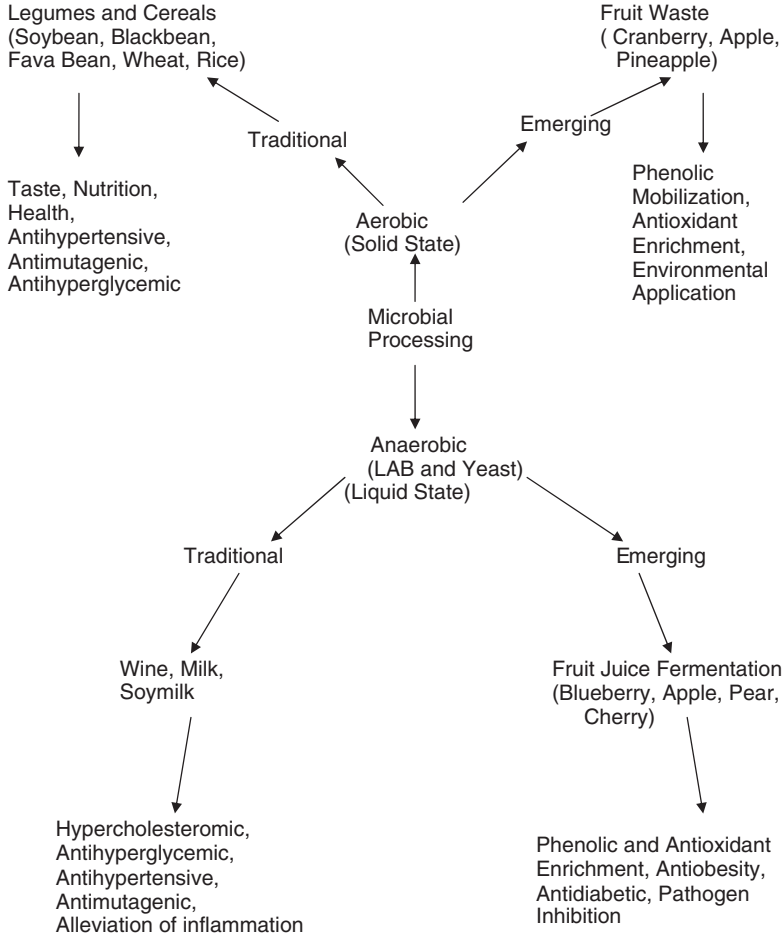

Figure 13.1. An overview of health benefits of traditional and emerging fermented foods.

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14 Postharvest Strategies to Enhance Bioactive Ingredients for Type 2 Diabetes Management and Heart Health

Dipayan Sarkar and Kalidas Shetty

14.1 INTRODUCTION

Fruits and vegetables have been an integral part of the human diet since the evolution of modern humans. Paleolithic humans obtained over 65% of their food energy from fruits and vegetables (Eaton et al., 1997). However, the adoption of organized agriculture and animal husbandry, particularly the increasing dependence on cereal grains as an energy source, reduced intake of fruits and vegetables to 20% or less of humans' total energy intake (Eaton et al., 1997). In many ancient literatures, consumption of fruits and vegetables were referred to as healthy dietary habits. The health benefit aspects of some fruits and vegetables and their uses to treat different diseases were described in the Ayurveda, or ancient Indian medical literature. Different fruits, such as apples, dates, pomegranates, and grapes, were frequently mentioned in the Bible. These fruits were associated with eternal life in ancient Egyptian and Sumerian cultures.

14.2 CHANGING DIETARY PATTERNS: A HISTORICAL PERSPECTIVE

With the advent of the Industrial Revolution and the development of modern agriculture, the dietary paradigm changed significantly. In the post–World War II era, global agriculture and food systems were concerned with yield and total food grain production in order to feed the increasing global population. The development of nitrogen fertilizers from the Haber–Bosch process, combined with agrochemicals and the advancement of modern plant breeding and biotechnology, helped us to reach sufficient food grain production to meet calorie needs linked to hunger in both developed and developing nations. Although global agriculture achieved a green revolution to meet calorie needs, with modern food processing

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and higher intake of refined calorie food products, it has slowly changed the qualitative aspects of our food, diet, and environment in many different ways. For example:

1. Monocropping and overdependence on grain production are a key part of the overall global food system.
2. Dietary consumption of carbohydrates (starches) has risen.
3. Replacement of old, high-quality cultivars with high-yielding, starch-based cultivars has resulted in a loss of biodiversity.
4. There has been a reduction in legumes, fruits, and vegetables in the daily diet.
5. With rapid urbanization has come a higher consumption of fast food with overconsumption of refined starch, sugar, and salt.
6. A change to a more sedentary lifestyle and a high-stress working environment has had physical as well as mental health consequences.

Overall, these changing patterns of diet and associated nutritional changes, combined with lifestyle changes, have caused an increasing incidence in some chronic diseases, including type 2 diabetes, cardiovascular disease, and cancer.

14.3 NONCOMMUNICABLE CHRONIC DISEASES: ERA OF NEW GLOBAL EPIDEMICS

Coronary heart disease (CHD), excess body weight (obesity), and type 2 diabetes are at the forefront of the growing global epidemic of chronic diseases. These diseases are interlinked and are major reasons behind cardiovascular disorders, which still represent the leading cause of mortality in developed and developing countries (Bisbal et al., 2010). Type 2 diabetes and cardiovascular disease have many common risk factors and are highly correlated with one another. "Metabolic syndrome" is a term used to refer to a range of interlinked physiological indicators that are powerful determinants of type 2 diabetes and cardiovascular disease and include hypertension, dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance, and, particularly, central obesity (Hanson et al., 2002) (Fig. 14.1). Cardiovascular diseases, diabetes, obesity, cancer, and respiratory conditions account for 59% of the 56.5 million deaths annually and 45.9% of the global burden of disease (Jaganath, 2008).

Worldwide, more than 220 million people have diabetes (WHO, 2010), with type 2 diabetes alone comprising 90% of the diagnosed incidences. The World Health Organization has projected that by the year 2025, the number of patients with type 2 diabetes in the world will reach about 300 million and diabetes deaths will double. In 2005, an estimated 1.1 million people died from diabetes; the actual number is probably much higher as cause of death is often recorded as heart disease or kidney failure. The latest estimation of diabetes patients in the North America and Caribbean region is 37.4 million (International Diabetes Federation, 2010). Type 2 diabetes has been considered to be a new epidemic in the American pediatric population, and a 33% increase in diabetes incidence and prevalence has been diagnosed during the past decade (Kaufman, 2002). The occurrence of type 2 diabetes is more prevalent in African-American, Mexican-American, Native-American, and Asian-American children and young adults.

Type 2 diabetes is a chronic metabolic disorder characterized by relative insulin deficiency due to impaired insulin production combined with peripheral insulin resistance

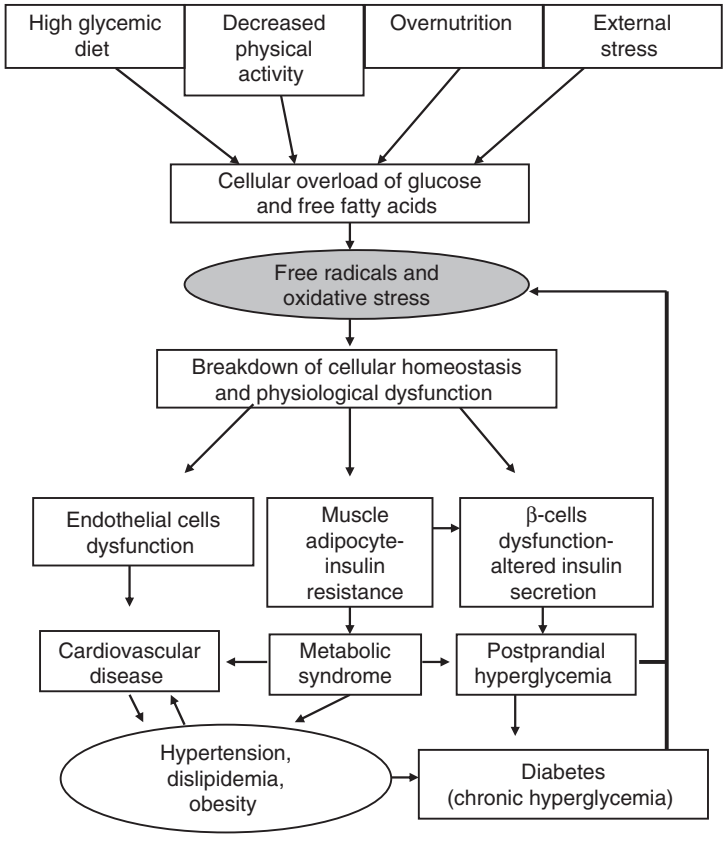

Figure 14.1. Diet and lifestyle induced oxidative stress and physiological dysfunction (hyperglycemia and cardiovascular disease) (adapted from Ceriello and Motz, 2004; Shetty et al., 2008).

(Green et al., 2003). The primary cause of fasting hyperglycemia is due to an elevated rate of basal hepatic glucose production in the presence of hyperinsulinemia, whereas postprandial (after a meal) hyperglycemia is due to the impaired suppression of hepatic glucose production by insulin and decreased insulin-mediated glucose uptake by muscles (DeFronzo, 1999). Patients with chronic diabetes experience different metabolic and physiological disorders.

Significant mortality due to both microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (heart attacks, stroke, and peripheral vascular disease) complications are common in many diabetic patients. Type 2 diabetes is the leading cause of blindness and end-stage renal failure in the United States (Klein, 1995). The risk of heart disease and stroke is two to four times more likely in persons with diabetes, and 50% of people with diabetes die due to cardiovascular disease. Diabetes, along with cardiovascular disease, has a significant socioeconomic impact on individuals, families, health systems, and countries. The World Health Organization reported that between 2006 and 2015, China will lose US$558 billion in national income due to heart disease, stroke, and diabetes. Type 2 diabetes and cardiovascular disease have genetic causes, but other factors such as obesity, physical activity, and food intake have been shown to significantly influence the pathophysiology of both diseases (Fitzgerald and Parekh, 2009).

14.4 HEALTHY DIET: "PREVENTION IS BETTER THAN CURE"

Across the globe there are many different dietary patterns, some of which promote health and others of which increase the risk of chronic disease (Kris-Etherton et al., 2002). Daily intake of food with high glycemic impact may increase the risk of obesity, type 2 diabetes, and cardiovascular disease by promoting excessive calorie intake, pancreatic β-cell dysfunction, dyslipidemia, and endothelial dysfunction (TörröneN et al., 2010). For human physiology, maintenance of glucose homeostasis is very important and failure of this strict hormonal control can result in a multisymptom disorder of energy homeostasis encompassing obesity, hyperglycemia, impaired glucose tolerance, hypertension, and dyslipidemia (Fig. 14.1; Hanhineva et al., 2010). Starch and sucrose are the most important dietary carbohydrates and most of them are digested, in the upper gastrointestinal tract, to monosaccharides, which are then absorbed into vascular circulation.

Healthy diet and proper nutritional compositions are key factors in the regulation of glucose metabolism. As nutrition itself is a tool in regulating glucose metabolism, some dietary patterns (e.g., the Mediterranean diet) are helpful for lowering the risk of CHD, diabetes, cancer, and cognitive impairment. The major characteristics of the Mediterranean diet which protect against diabetes include a high intake of fiber, high intake of vegetable fat, a low intake of *trans* fatty acids, and a moderate intake of alcohol (Martinez-Gonzalez et al., 2008). Although this dietary pattern has a relatively high total fat content, it is low in saturated fatty acids and rich in monounsaturated fatty acids from olive oil, which improves lipid profile and glycemic control in people with diabetes. Many recent studies reported that adherence to a Mediterranean diet is associated with reduced cardiovascular disease and total mortality (Martinez-Gonzalez et al., 2002; Trichopoulou et al., 2003; Panagiotakos et al., 2004).

Diets rich in soluble and insoluble fiber have different health benefits, such as inducing satiety, reducing total energy intake and adiposity, improving glycemic control, reducing blood lipids, and preventing constipation, diverticuli, and other disorders of the gastrointenstinal tract (McIntosh and Miller, 2001). Consumption of fruits, vegetables, whole grains, beans, and other foods rich in soluble and insoluble fiber has been suggested to reduce the risk of CHD, diabetes, and cancer.

14.4.1 Fruits and vegetables: from garden of eden to modern horticulture

Fruits and vegetables are rich sources of a wide range of vital micronutrients, vitamins (provitamin A carotenoids, vitamin C, and folate), phytochemicals (non-provitamin A carotenoids and polyphenols), and fiber (Amiot and Lairon, 2010). All of these components are probably involved in producing different beneficial effects for human health. Fruits and vegetables are generally low-energy foods, and, if substituted for high-energy, fatty, and sugary foods, can prevent obesity-related chronic diseases such as diabetes (Berrino and Villarini, 2010). Nutritional recommendations in the majority of developed countries encourage increased consumption of fruits and vegetables. Extensive campaigns to achieve this (such as the United States' "five-a-day" campaign) are reported to be successful in many areas, but consumption of fruit and vegetables is still below the recommended level in many countries. Daily intake of fresh fruit and vegetables in an adequate quantity (400–500 g/ day) is recommended to reduce the risk of cardiovascular disease, stroke, and high blood pressure (Jaganath, 2008). In the United Kingdom, for example, for optimum

health it is advised to consume five portions of fruit and vegetables (each comprising at least 80 g) on a daily basis (Williams, 1995).

Many epidemiological studies indicate an inverse relationship between the consumption of fruits and vegetables and the incidence of cardiovascular disease. Dauchet et al. (2006) reported that the risk of CHD was decreased by 4% for each additional portion per day of fruit and vegetable intake. In another study, Bazzano et al. (2003) found that higher frequency of fruits and vegetables in daily diets reduced stroke incidence, stroke mortality, ischemic heart disease mortality, and cardiovascular disease mortality. The associations between daily consumption of green-yellow vegetables and fruits with a lower risk of total stroke, intracerebral haemorrhage, and cerebral infarction mortality have been found in Japanese men and women after an 18-year follow-up period (Sauvaget et al., 2003).

In order to asses the role of fruits and vegetables in the prevention of type 2 diabetes, several studies have been conducted in past two decades. Ford and Mokdad (2008) reported that a higher consumption of fruit and vegetables appeared to prevent type 2 diabetes in women, but no association was found in men. They observed that the risk of disease is diminished by almost 40% in women consuming five portions a day of fruits and vegetables. A study with Finnish men and women also found that a higher intake of fruits, especially berries (>136 g/day), diminished the risk of diabetes by 30%, but no effect was observed for vegetables (Montonen et al., 2005). Villegas et al. (2008) found an inverse relationship between vegetable intake and type 2 diabetes incidence in Chinese women. Similarly, Liu et al. (2004) observed that a high intake of fruits and vegetables reduces the risk of type 2 diabetes. There are multiple biological mechanisms involved for the beneficial effect of the fruits and vegetables on diabetes risk. Other than high fiber content, low energy intake, and low glycemic load, fruits and vegetables are also rich in antioxidant enzymes, vitamins, magnesium, potassium, plant proteins, and other individual phytochemicals (Bazzano et al., 2003). Fruits and vegetables are beneficial for lowering cardiovascular disease and diabetes prevalence in a variety of ways (dietary guidelines prepared by Secretaries of Health and Human Services and Agriculture, 2005; http://www.longislanddiabetes.org/Microsoft%20Word%20-%20Chronic%20Disease%20Fact%20Sheet%20for%20ADA.pdf):

1. Fruits and vegetables provide nutrients such as fiber, folate, potassium, and carotenoids, as well as other phytochemicals that may directly reduce cardiovascular disease risk.
2. They also reduce other diet-related risk factors, such as blood pressure, hyperlipidemia, and diabetes.
3. Consumption of fruits and vegetables may lead to a reduced intake of saturated fat and cholesterol.

14.5 BIOACTIVE INGREDIENTS

Bioactive ingredients are defined as essential and nonessential compounds (e.g., vitamins and polyphenols) that occur in nature and are part of the food chain, and can be shown to have an effect on human health (Biesalski et al., 2009). They are also referred as "extra-nutritionals" (Kitts, 1994) or "nutraceuticals" (coined in 1979; DeFelice, 1992) that typically occur in small quantities in plant, animal, and microbial food products and can be found as water-soluble forms as well as in lipid-rich foods. The increasing interest in bioactive compounds lies in the emerging knowledge from epidemiological studies that a specific

diet or a component of the diet can alter the risk of chronic diseases including cancer, cardiovascular disease, and diabetes (Biesalski et al., 2009).

Phytochemicals are structurally diverse bioactive compounds found in fruits, vegetables, grains, and other plant-based foods. More than 45,000 phytochemicals have been identified, and they are classified into four major groups: terpenoids, phenolics and polyphenolics, nitrogen-containing alkaloids, and sulfur-containing compounds (Crozier et al., 2006). Phytochemicals are secondary metabolites, providing unique survival and adaptive strategies to plants by acting in defense against abiotic stresses such as UV-B irradiation, temperature extremes, low water potential, or mineral deficiency, as well as biotic stress, but at the same time they also provide a beneficial impact on human health. Phytochemicals with antioxidant properties have been investigated most extensively, as increased intake of these can alter the risk of chronic diseases including cancer and cardiovascular disease. Different classes of phytochemicals have been identified as having preventative effects against specific diseases, mainly at very early stages of disease development.

14.6 DIETARY POLYPHENOLS: IMPACT ON HUMAN HEALTH

Among phytochemicals, polyphenols, including flavonoids, phenolic acids, proanthocyanidins, and resveratrol are a large and heterogeneous group of chemicals in plant-based foods such as tea, coffee, wine, cocoa, cereal grains, legumes, including soy, and vegetables and fruits, including berries. They are the most abundant antioxidants in the human diet. Structurally, these compounds are designed with an aromatic ring carrying one or more hydroxyl moieties. The average estimated intake of dietary polyphenols is approximately 1 g/day (Ovaskainen et al., 2008). Many *in vitro* and *in vivo* studies on polyphenols showed that they posses anti-inflammatory, antioxidative, chemopreventive, and neuroprotective activities. Recent studies indicate that dietary polyphenols also influence glucose and lipid metabolism (Hanhineva et al., 2010).

Most dietary polyphenols are metabolized by colonic microbiota before absorption, but a smaller amount can be absorbed directly from the upper gastrointestinal tract (Selma et al., 2009). Gut bacteria modulate the biological activity of polyphenols by various mechanisms, and this metabolic process is a prerequisite for absorption. The mode of action and systemic effects of dietary polyphenols largely depend on synergistic action and are affected by other bioactive constituents present in the diet (Liu, 2003). Dietary polyphenols and their metabolites may influence digestion, absorption, and metabolism of dietary carbohydrates such as starch and sucrose.

14.6.1 Role of polyphenols in glucose metabolism

14.6.1.1 Alpha-amylase and α-glucosidase inhibition

Dietary α-amylase and α-glucosidase are the key enzymes responsible for digestion of dietary carbohydrates to glucose in humans. A wide range of polyphenols has been shown to inhibit α-amylase and α-glucosidase activities *in vitro* (Kim et al., 2000; Grover et al., 2002; McCue and Shetty, 2004; McCue et al., 2005). Phenolic compounds are known to interact with protein structure and can inhibit enzymatic activity (Dawra et al., 1988). The group of polyphenols that show inhibitory activity are flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones, and isoflavones), phenolic acid, and tannins (proanthocy-

anidins and ellagitannins). Many foods are rich in phenolic composition, including berries (strawberries, raspberries, blueberries, and blackcurrants) (McDougall and Stewart, 2005; Cheplick et al., 2007, 2010; Pinto et al., 2008, 2010), vegetables (pumpkin, beans, maize, pepper, and eggplant) (McCue et al., 2005; Kwon et al., 2007a,b, 2008a), colored grains such as black rice (Yao et al., 2009), green and black tea (Koh et al., 2009), and red wine (Kwon et al., 2008b), and all have shown significant α-glucosidase and α-amylase inhibitory activities in many *in vitro* studies.

Cheplick et al. (2007) reported high α-glucosidase inhibitory activity for a yellow raspberry cultivar among red, black, and yellow raspberries, suggesting that the α-glucosidase may be influenced more by specific anthocyanins rather than the actual amount of the overall total plant phenolics. Apostolidis et al. (2007) reported that cranberry-enriched cheese had the highest α-glucosidase and α-amylase inhibitory activities among herb-, fruit-, and fungal-enriched cheeses by *in vitro* assays.

Pinto et al. (2008) studied the potential effects on the *in vitro* inhibition of α-amylase and α-glucosidase enzymes from different Brazilian strawberry cultivars. They found that strawberries had high α-glucosidase and low α-amylase inhibitory activities, suggesting these fruits as good sources for potential management of hyperglycemia linked to type 2 diabetes as a part of an overall diet. Apostolidis et al. (2006) reported that the combinations of cranberry with oregano, which had higher rosmarinic acid content, contributed to the high antioxidant activity and total phenolic content in the extracts, suggesting potential relevance for using these are whole plant extracts as supplements for management of type 2 diabetes and related macrovascular complication such as hypertension.

14.6.1.2 Glucose transport

Polyphenols also influence glucose transporters and thus mediate intestinal absorption of glucose. Phenolic acids and several flavonoids including chlorogenic, ferulic, caffeic, tannic acids (Welsch et al., 1989), quercetin monoglucosides(Cermak et al., 2004), tea catechins (Kobayashi et al., 2000), and naringenin (Li et al., 2006) have been found to inhibit Na^+-dependent SGLT1-mediated glucose transport. GLUT2-dependent glucose transport was also inhibited by quercetin, myricetin, apigenin, and tea catechins (Song et al., 2002).

14.6.1.3 Blood glucose response

Different studies using animal models have also proven the impact of polyphenols on postprandial blood glucose response. Anthocyanin extract and diacylated anthocyanin from purple sweet potatoes reduced blood glucose and insulin responses to maltose administration in rats (Matsui et al., 2002). Reduced plasma glucose level in mice after administration of maltose or glucose with a crude acerola polyphenol fraction was observed, suggesting inhibition of α-glucosidase and intestinal glucose transport (Hanamura et al., 2006). Johnston et al. (2002) have found that apple extract containing polyphenols such as chlorogenic acid and phloridzin can significantly lower mean plasma glucose concentration in healthy humans 15 minutes after ingestion. Similarly, berries also significantly decreased the peak glucose increment by reducing the rate of sucrose digestion or absorption from the gastrointestinal tract (TörröneN et al., 2010). Instant black tea can attenuate late postprandial glycemia by an elevated insulin response following the stimulation of pancreatic β cells (Bryans et al., 2007).

14.6.1.4 Beta-cells dysfunction

Prolonged hyperglycemia and hyperlipidemia leads to the dysfunction of the pancreatic β-cells, resulting in autocrine insulin resistance, impaired insulin secretion, decreased expression of genes involved in insulin production, and, finally, decrease in β-cell mass caused by apoptosis (Hanhineva et al., 2010). Soybean isoflavonoids have been shown to have a positive impact on β-cell function. Isoflavonoids, genistein, and daidzen preserved the insulin production by β cells in mice (Choi et al., 2008). In another study, epigallocatechin gallate and rutin elevated intracellular ATP, suggesting that the increase in insulin secretion is mediated by enhancing the normal, glucose-induced insulin secretion that is dependent on ATP concentrations (Qa'dan et al., 2009).

14.6.1.5 Glucose uptake

Polyphenols may also influence the tissue uptake of glucose by stimulating peripheral glucose uptake in insulin-sensitive and non-insulin-sensitive tissues. Chlorogenic acid, ferulic acid, aspalathin, resveratrol, kaempferol, and quercetin have shown positive response through increased glucose uptake in different studies (Park et al., 2007; Fang et al., 2008; Prabhakar and Doble, 2009). Plant-based foods and plant extracts also can enhance glucose uptake. Tea extracts, fruit juice extract of bitter melon, and cinnamon extracts were shown to stimulate glucose uptake into muscle cells (Anderson et al., 2004; Cummings et al., 2004; Pinent et al., 2004).

14.6.1.6 Prevention of type 2 diabetes development

Among important strategies of food design, one of the most important is to understand the beneficial effects of polyphenols to prevent of type 2 diabetes. Whole grain and high coffee consumption was associated with lower risk of type 2 diabetes (Pereira et al., 2006). Similarly, apples and tea also proved to have a positive impact in lowering type 2 diabetes incidences in middle-aged women (Song et al., 2005). In another study, apples and berries were the most important contributors lowering the risk of type 2 diabetes in Finnish men and women (Knekt et al., 2002).

14.6.2 Polyphenols and cardiovascular disease

Many epidemiological studies have shown the beneficial effect of dietary polyphenols in reducing the risk of cardiovascular disease. Arts and Hollmann (2005) reported that catechins, flavones, and flavonols can significantly reduce the risk of cardiovascular disease. Increasing amounts of evidence are confirming that other than their antioxidant properties, these compounds have additional cardioprotective functions by improving plasma lipid profiles and reducing inflammation (Zern and Fernandez, 2005). Polyphenols may provide multifunctional roles by reducing cholesterol absorption by upregulating hepatic mRNA abundance for the LDL receptor and by affecting apo B secretion rates [microsomal triglyceride transfer protein (MTP) and acyl coenzyme A] (Amiot and Lairon, 2010). Procyanidins are also capable of inhibiting cholesterol esterification and intestinal lipoprotein secretion in appropriate nutritional amounts (Vidal et al., 2005). Flavonoids participate in the regulation of cellular function through inhibition of prooxidant enzymes (xanthine oxidase, nicotinamide adenine dinucleotide phosphate oxidase [NADPH], lipoxygenases),

induction of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione S-transferase), and inhibition of the redox-sensitive transcription factors (Manach et al., 2004).

14.7 PHENOLIC BIOSYNTHESIS: BIOLOGICAL MECHANISM TO IMPROVE DIETARY POLYPHENOLS IN PLANT MODELS

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways (Shetty, 1997). The first step in the synthesis of phenolic compounds is the commitment of glucose to the pentose phosphate pathway (PPP), converting glucose-6-phosphate irreversibly to ribulose-5-phosphate. This first committed step in the conversion to ribulose-5-phosphate is carried out by glucose-6-phosphate dehydrogenase (G6PDH). The conversion to ribulose-5-phosphate also produces reducing equivalents (NADPH) for cellular anabolic reactions. The pentose phosphate pathway also generates erythrose-4-phosphate, which, along with phosphoenolpyruvate from glycolysis, is channeled to the shikimate pathway to produce phenylalanine, which is directed through the phenylpropanoid pathway to produce phenolic compounds. Many phenylpropanoid compounds, such as flavonoids, isoflavonoids, anthocyanins, and polyphenols, are induced in response to wounding, nutritional stress, cold stress, high visible light, and UV radiation in plants (Beggs et al., 1987; Graham, 1991; Christie et al., 1994).

Shetty (1997) proposed a model that a proline-mediated pentose phosphate pathway could stimulate both the shikimate and phenylpropanoid pathways, and therefore, the modulation of this pathway could lead to the stimulation of phenolic phytochemicals in plants (Fig. 14.2). Using this model proline, proline precursors and proline analogs were effectively utilized to stimulate total phenolic content in plants (Kwok and Shetty, 1998; Yang and Shetty, 1998). It was also proposed that demand for NADPH for proline synthesis during microbial interaction and proline analog treatment may increase cellular NADP+/NADPH ratio, which should activate G6PDH. The proline analog azetidine-2-carboxylate (A2C) is an inhibitor of proline dehydrogenase and therefore tolerance to the analog could stimulate proline synthesis, which drives the demand for NADPH (Elthon and Stewart, 1984). Another analog, hydroxyproline, is a competitive inhibitor of proline for incorporation of proteins. Either analog at low levels should deregulate proline synthesis from feedback inhibition, therefore allowing proline synthesis. As a consequence, deregulation of the pentose phosphate pathway may drive metabolic flux toward erythrose-4-phosphate for biosynthesis of shikimate and phenylpropanoid metabolites. At the same time, proline serves as a reducing equivalent, instead of NADH for oxidative phosphorylation (ATP synthesis) in mitochondria (Shetty and McCue, 2003; Shetty, 2004).

This model has been proposed for the mode of action of phenolic metabolites based on the correlation between stress-stimulated phenolic biosynthesis and stimulation of antioxidant enzyme response pathways in plants. Within the plant system model, acid, exogenous phenolic, proline analogs and precursor combinations, and microbial elicitors were used to stimulate phenolic biosynthesis and key enzyme responses (Shetty, 2004). Proline/G6PDH correlations during phenolic response were also associated with phenolic content, potential polymerization of phenolics by glutathione peroxidase (GPx), and antioxidant activity based on the free radical scavenging activity of phenolics and superoxide dismutase (Bowler et al., 1994; McCue et al., 2000; McCue and Shetty, 2002).

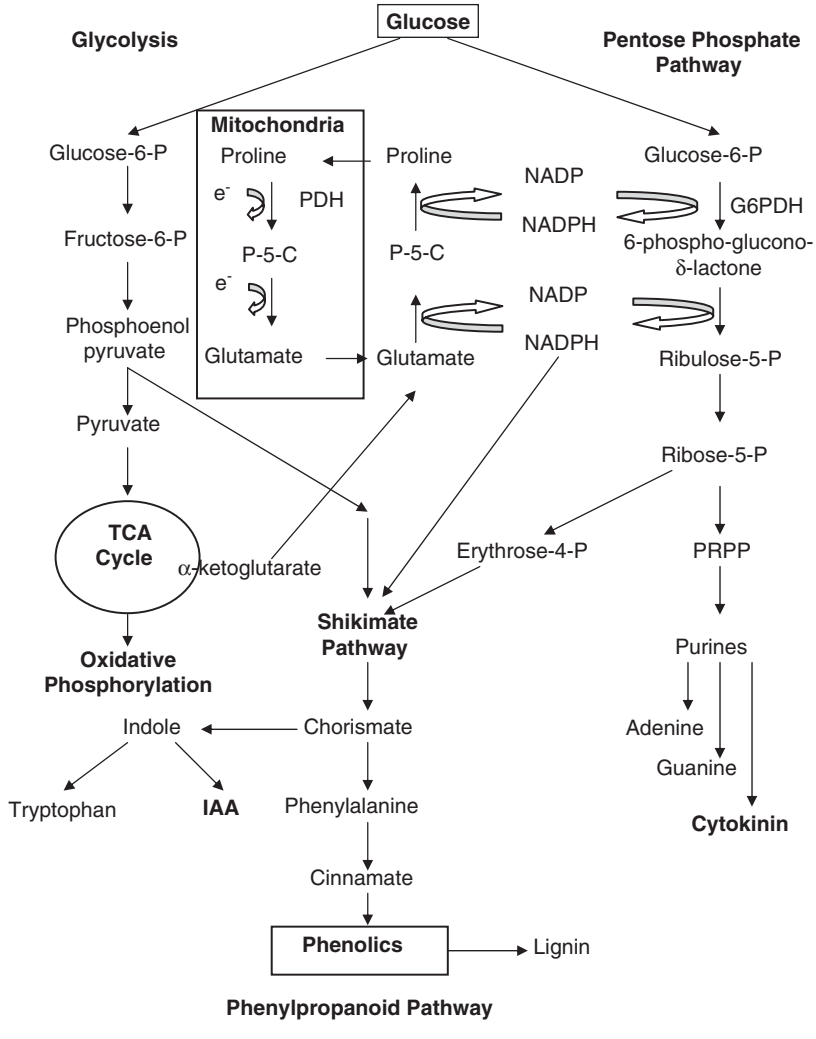

Figure 14.2. Biosynthesis of phenolic compounds and proline-associated pentose phosphate pathway regulation in plants (adapted from Shetty, 1997, 2004).

The synthesis of phenolic metabolites and stimulation of antioxidant response pathway would be able to minimize the oxidation-induced damage within tissues where it occurs. Phenolic antioxidants can behave like antioxidants by trapping free radicals in direct interactions or scavenge them through a series of coupled, antioxidant enzyme defense system reactions. A coupled enzymatic defense system could involve low-molecular-weight antioxidants such as ascorbate, glutathione (GSH), α-tocopherol, carotenoids, and phenylpropanoids, in conjunction with several enzymes such as superoxide dismutase, catalase, peroxidases, glutathione reductase, and ascorbate peroxidase (Bowler et al., 1994; Rao et al., 1996; Pinhero et al., 1997). So with the above-mentioned rationale, it is possible to develop different dynamic strategies to harness the benefits of phytochemicals for the development of functional foods and nutraceuticals to counter chronic diseases in pre- and postharvest stages of fruits and vegetables.

14.8 POSTHARVEST STRATEGIES TO IMPROVE BIOACTIVE INGREDIENTS IN FRUITS AND VEGETABLES

Globally, consumers are becoming increasingly health-conscious, and horticultural produce rich in bioactive ingredients is important for providing healthy, fresh foods. As they provide an optimal mixture of phytochemicals, research into rational design of nutraceuticals and functional food has highlighted the structure-function linked healthy compounds found in fruits and vegetables (Shetty and McCue, 2003; Kwon et al., 2007a,b; Shetty et al., 2008). It is important to understand the relevance and influence of maturity, agronomic practices, postharvest handling, processing, and storage conditions on the biochemical characteristics of fruits and vegetables. Many earlier investigations were mostly focused on the physical characteristics of fruits and vegetables to determine the quality parameters. But as bioactive phytochemical compounds of fruits and vegetables become an increasingly important factor, it is of great interest to evaluate the changes of this biochemical and metabolic quality parameter during postharvest storage of fresh fruits and vegetables (Ayala-Zavala et al., 2007). During postharvest storage and handling, several factors such as species, variety, microbial load, presence of pests, temperature, light condition, humidity, radiation exposure, packaging, and chemical treatments significantly influence biochemical properties and rate of the deterioration of fruits and vegetables (Bengtsson and Hagen, 2008).

Although in postharvest stages fruits and vegetables are detached from mother plants, they continue to respire and have metabolic activities. The longevity, taste, and changes in chemical compositions of fruits and vegetables are determined by a series of biotic and abiotic factors during pre- and postharvest stages. The rule of thumb is to check respiration, as the higher the rate of respiration means, the faster the rate of deterioration of fruits and vegetables, and this can have consequences for regulation of redox function and metabolism associated with redox biology. This postharvest status is linked to eventual phytochemical content relevant for both preservation and human health.

14.8.1 Temperature

Lower temperatures generally slow down respiration rates, ripening, and senescence processes, as well as the growth of pathogenic and deteriorative microorganisms and thus keep fruits and vegetables healthy during storage. Some studies reported that cold storage (0 and 1°C) did not affect β-carotene, α-carotene, and total carotenoids of carrots during storage (Kidmose et al., 2006; Koca and Karadeniz, 2008), whereas some authors observed minor degradation of carrot carotenoids during cold storage (Kopas-Lane and Warthesen, 1995; Howard et al., 1999). Ayala-Zavala et al. (2004) reported that total anthocyanin content in strawberries increased gradually at 10°C, but it decreased at 0 and 5°C after 5 days of storage. However, total phenolic compounds stayed at a constant value during the storage period of 13 days. Leja et al. (2003) found an increase in total phenols of Jonagold apples after 120 days of storage at 0°C, and an increase of phenolics positively correlated with increase of ethylene in the same period. MacLean et al. (2006) observed a small decrease in anthocyanins and increase of chlorogenic acid, but no change in overall content of phenolic compounds in red delicious apples during 120 days of cold storage. Goncalves et al. (2004) studied the bioactive compound of four different cultivars of cherry during postharvest storage. They found that phenolic contents generally decreased with storage at

1–2°C and increased with storage at 15 ± 5°C. Significant increases in phenolic content were observed in Hayward kiwifruit after 6 months' storage at 0°C followed by a week at ambient temperature (25°C) (Tavarini et al., 2008).

14.8.2 Light and oxygen

Light conditions and oxygen concentration also have a significant influence on quality during the storage period of fruits and vegetables. Phenolic content of fruits and vegetables generally increases with elevated light or UV irradiation. Tenfold higher concentration of quercetin glycoside content was observed in a sun-exposed cluster of Pinot Noir grapes (Price et al., 1995). Higher anthocyanin and quercetin content was found in sun-exposed portions of Jonagold and Elster apples (Lancaster et al., 2000). Awad et al. (2001) reported that anthocyanin and quercetin compounds were higher in sun-exposed apples, but other major phenolic compounds such as catechins, phloridzin, and chlorogenic acid were not different between sun-exposed and shaded apples. High correlation between phenolic compounds and oxygen concentration (>21 kPa) was observed in many fruits during storage. Zheng et al. (2008) reported that a high-oxygen atmosphere increased the phenolic content of blueberries during storage. The oxidative stress due to higher oxygen concentration might be the reason behind the higher phenolic content, as stress-induced conditions stimulate pentose phosphate, shikimate, and phenylpropanoid pathways.

14.8.3 Chemical treatment and natural compounds

Other than the physical conditions, uses of chemical treatments including natural products or antioxidant stimulators are increasingly gaining importance in the postharvest storage of fruits and vegetables. Sharma et al. (2010) reported that Hexanal vapor and 1-MCP (Methylcyclopopene) treatments increased firmness, superoxide dismutase, and ascorbate peroxidase activity in sweet cherries. They also observed that levels of anthocyanin or phenolic component were either enhanced or maintained during 30 days of storage. Similarly, Chen et al. (2010) found that 1-MCP vacuum infiltration treatment significantly improved the physiological quality response of Suli pears in cold storage. Ayala-Zavala et al. (2005) observed continuous decrease in anthocyanin content of strawberry fruit after treatments with natural antimicrobials (methyl jasmonate, ethanol, and combinations of both). But total phenolic compounds and antioxidant capacity increased sharply during the first 5 days at 7.5°C.

Natural antioxidant treatments are generally used to check biochemical deterioration such as enzymatic browning in fruits. Ascorbic acid is the most widely used antioxidant, whereas cystein, *N*-acetyl cysteine, calcium salts, citric acid, and some other organic acids are also used to increase the shelf life of the fruit. Antioxidant dipping treatment (1% ascorbic acid + 1% citric acid) increased ascorbic acid content and total polyphenols content in freshly cut apples (Cocci et al., 2006). In another study, Robles-Sanchez et al. (2009) reported higher total phenolic content in freshly cut mango cubes after antioxidant dipping treatments. Sun et al. (2010) found that ascorbic acid, along with 1% chitosan solution, increased superoxide dismutase, catalase activity, and ascorbic acid and glutathione content in the pulp of treated litchi.

14.9 PHENOLIC-LINKED ANTIOXIDANT ACTIVITY DURING POSTHARVEST STAGES IN FRUITS AND RELEVANCE FOR TYPE 2 DIABETES

Postharvest preservation of fruits depends to a significant extent on the phenolic-linked antioxidant activity during storage. In a study with four different cultivars of apples, Adyanthaya et al. (2009) found initial increase of superoxide dismutase activity during storage (5°C for 120 days) in well-preserved cultivars. The higher superoxide dismutase activity correlated with higher phenolic content and high free radical scavenging-linked antioxidant activity. In another study Adyanthaya et al. (2010) observed that during 120 days of storage, apple cultivars with higher phenolic content were associated with enhanced postharvest preservation and also had high α-glucosidase inhibitory activity relevant for management of hyperglycemia. They suggested a probable role of phenolic-enriched apples in the modulation of postprandial glucose increase and in the reduction of the risk of developing type 2 diabetes.

Barbosa et al. (2010) also found positive correlation between phenolic content and α-glucosidase inhibitory activity in aqueous and ethanol extracts of 10 different apple cultivars after harvest. Red delicious and honeycrisp cultivars had higher phenolic content and high total antioxidant activity. They found high phenolic content, high antioxidant activity, and high α-glucosidase inhibitory activity in the peel extracts compared to pulp extracts. The aqueous pulp extracts showed higher α-amylase inhibitory activity compared to that of peel extracts. The main phenolic compounds found in peel extracts were quercetin derivatives, protocatechuic acid, and chlorogenic acid, whereas pulp extracts had quercetin derivatives, chlorogenic acid, and *p*-coumaric acid. In another postharvest study, a similar correlation between phenolic content and α-glucosidase inhibition in both peel and pulp extracts of six different apple cultivars during 6 months of storage was observed (Sarkar et al., unpublished results). Apples treated with 1-MCP significantly maintained firmness, total phenolic content, total antioxidant activity, α-glucosidase, and α-amylase inhibitory activity in both peel and pulp extracts over the storage period. The effect of the chemical treatment (1-MCP) was more prominent in peel compared to the pulp extracts. All of these *in vitro* studies showed a strong association between phenolic content and α-glucosidase inhibitory activity and the promise of the apple as a dietary component to counter the risk of type 2 diabetes development and to manage diabetic complications.

In another *in vitro* study with different pear cultivars, similar enzyme inhibitory activity was observed (Sarkar et al., unpublished results). Pear cultivars from Oregon and Massachusetts, including red Anjou, green Anjou, Bartlett, and Starkrimson showed higher phenolic content, high antioxidant activity and high α-glucosidase inhibitory activity in both peel and pulp extracts. High α-amylase inhibitory activity was observed in aqueous pulp extracts of these pears.

Similarly, different sweet cherry and tart cherry cultivars also showed significant potential for type 2 diabetes management. Out of 22 different cherry cultivars, many (Montmorencey, Jubileum, Northstar, Baleton) showed significantly higher phenolic content, high antioxidant activity, and α-glucosidase inhibitory activity. High α-glucosidase inhibition and low α-amylase inhibitory activity was observed in almost all cherry cultivars (Sarkar et al., unpublished results). Therefore, with this interesting finding, cherries also offer the potential for good postprandial blood glucose management without the common

side effects associated with high α-amylase inhibition. Fruits (apples, pears, and cherries) and cultivars with high phenolic content not only have the potential to control postprandial hyperglycemia but also have the potential to counter any cellular redox imbalances and possibly prevent diabetic complications through free radical scavenging-linked antioxidant activity. Further clinical studies based on animal models along with epidemiological studies are necessary to further validate the strong biochemical rationale and potential of these *in vitro* studies for type 2 diabetes and chronic cardiovascular disease management.

14.10 FUTURE DIRECTION OF RESEARCH: WHEN FUNCTIONAL FOOD AND DIET BECOME "PANACEA"

An integrated and systematic approach is needed to counter the global epidemic of noncommunicable diseases. People generally eat meals mixing different foods, giving several nutrients a chance to interact with one another. Not only are the macronutrients, minerals, vitamins, and other bioactive components and biochemicals present in foods responsible for triggering host-linked metabolic responses but microbes including several gut bacteria are also interacting in this complex process of metabolism. It is important to understand overall system biology to design functional foods to provide a sustainable chronic disease management option. Different factors in different growth and developmental stages play significant roles in dictating the phytochemical composition of fruits and vegetables and their beneficial effects on health. To harness the maximum benefits of fruits and vegetables in disease management, we have to consider and optimize many determining factors involved in metabolic responses of plant growth and development coupled with their biotic and abiotic responses, especially in fruiting stages to postharvesting stages. Furthermore, various stages of processing have to be coupled with *in vivo* and *in vitro* studies to optimize health-relevant functionality before they can be used in effective education-linked delivery based on specific human health needs (Fig. 14.3).

14.10.1 Stage 1: physiology and growth during germination to maturity

Initial growth and germination of seeds and root stocks depends on several physiological and biochemical mechanisms linked to redox function due to demand for oxygen for diverse metabolic adjustments. Seed treatment with natural and synthetic chemicals, use of growth regulators or biostimulators in cuttings, and agronomic manipulation such as mulching all significantly alter the phytochemical content of fruits and vegetables associated with metabolic adjustments. During developmental stages, several agronomic and horticultural practices also influence the chemical composition of fruits and vegetables, and proper management options can help to develop food with higher health-relevant bioactive compounds. Biotic and abiotic stresses are the most crucial factors regarding phytochemical synthesis associated with metabolic adjustment in diverse stages and especially later, in postharvest stages. For the development of fruits and vegetables with high bioactive components, it is important to manipulate abiotic and biotic stresses (temperature, UV irradiation, water, salinity, pathogens, and insects) to stimulate secondary metabolite synthesis, including that of polyphenols, relevant for both stress-linked adjustment and also crossing over into health benefits. During maturity or harvest, treatment with natural antioxidant

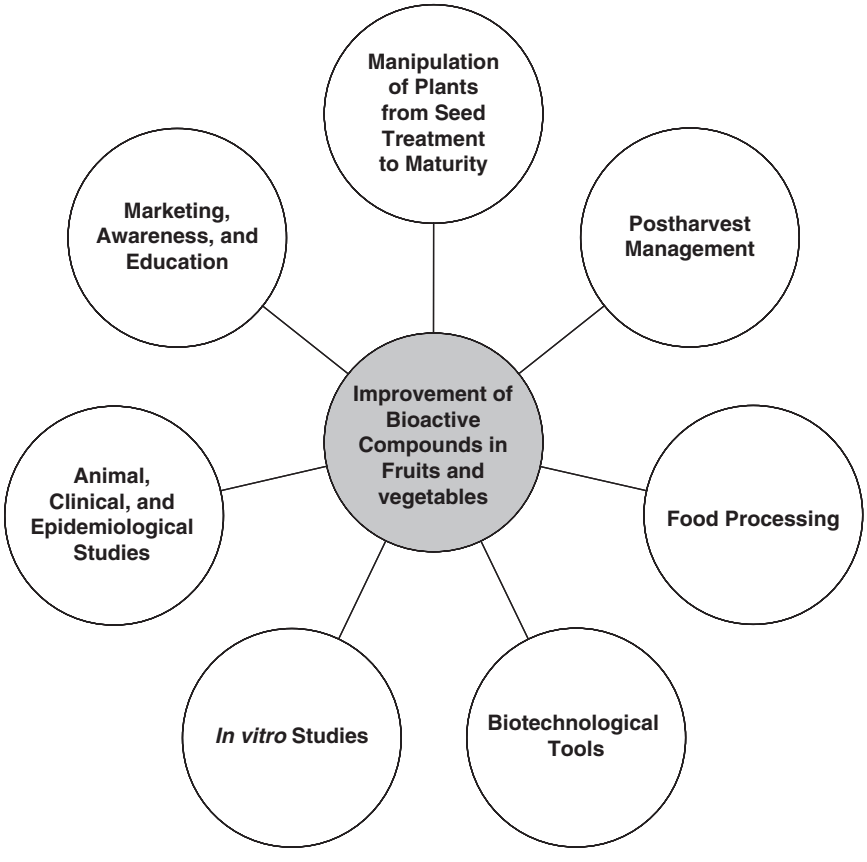

Figure 14.3. Different sustainable approaches to improve bioactive ingredients in fruits and vegetables for countering noncommunicable diseases including type 2 diabetes and cardiovascular diseases.

stimulators or other chemicals could also improve the longevity, keeping quality, edibility, functionality, and health-relevant phytochemical composition of fruits and vegetables.

14.10.2 Stage 2: postharvest management

Storage condition, processing, and packaging significantly influence the biochemical compositions of fruits and vegetables during postharvest stages. We should critically control all of these processes to improve and maintain health-relevant bioactive compounds linked to specific health conditions. Research should focus on improving selected bioactive compounds with specific targeted health benefit profiles in specific fruits and vegetables with innovative and sustainable technologies available for postharvest management such as natural stress modulators from diverse food-grade biological systems.

14.10.3 Stage 3: food processing

During processing of fruits and vegetables, the potential for loss of bioactive properties is high, and strategies should be developed to minimize such losses. Additives and

preservatives significantly change the chemical composition of food. It is important to find out the chemical interactions and synergistic effects of different natural chemicals during processing in canned, processed, and stored foods. Different medicinal or natural plant products such as extracts from berries, medicinal plant extracts such as sugar and salt supplements, and natural chemicals with high phenolic content that can be antioxidant and antimicrobial can be helpful to enrich the processed foods for better preservation and also to retain bioactive components relevant for chronic disease management.

14.10.4 Stage 4: biotechnological tools

New developments in plant biotechnology can also help to develop fruits and vegetables with high bioactive compounds. It is important to understand the exact biochemical control points of the biosynthetic pathway of the stress-inducible bioactive compounds, as primary targets are the controlling enzymes involved in their biosynthesis. Adequate balance between the specific induction of target enzymes involved in the biosynthesis of the desired phytochemicals and the nonactivation of those catabolic enzymes involved in the degradation of phytochemicals is the key for genetic manipulation. With modern genetic engineering or tissue culture technology, it is possible to produce high-value fruits and vegetables with proper metabolic adjustments that maximize plant growth, development, and postharvest preservation, while retaining bioactive compounds for health benefits.

14.10.5 Stage 5: *in vitro* studies

Through *in vitro* studies we can screen species and cultivars of fruits and vegetables with better health-relevant phytochemical composition to treat chronic diseases by targeting specific enzymes relevant for a specific health condition. Research regarding the biochemical mechanism of specific compounds and specific whole food profiles is also important for current and future development of novel foods and food ingredients targeted for specific health benefits, especially in the management of chronic disease conditions linked to metabolic syndrome such as type 2 diabetes and cardiovascular disease.

14.10.6 Stage 6: animal, clinical, and epidemiological studies

In vitro studies should lead to the biochemical rationale for proper human clinical studies, possibly with intervening animal (rats, mice, or other mammalians) model studies to further validate the need for more expensive clinical studies. Through this validation approach it is not possible to screen a wide variety of foods and food systems to prove the beneficiary effect of certain whole food bioactive profiles or specific phytochemicals to counter diseases such as type 2 diabetes and cardiovascular disease. Epidemiological studies with specific race, gender, or age groups are also important to find out the effect of dietary patterns and their benefit against these chronic diseases. Integrating the knowledge of postharvest and *in vitro* studies into the *in vivo* model could be critical for the success of such research. In clinical studies, researchers should also consider the effect of both exogenous and endogenous probiotic microbes in our health, as any bioactive profiles will be subject to alterations by gut bacterial consortium and will also be changed by the intake of specific foods and food components.

14.10.7 Stage 7: marketing, awareness, and education

There is a need to develop proper education and marketing strategies so that the benefits of functional food can reach the consumers. Phytochemical composition and antioxidant properties of fruits and vegetables or processed products should be labeled on the packages, which could help to increase the consumer's knowledge about the benefits of these products. Health awareness and educational programs related to diet and health should be initiated by the public and private agencies in schools, colleges, and communities, in collaboration with health care and health delivery sectors to achieve the maximum benefit from enhanced intake of fruits and vegetables with better bioactive profiles for managing chronic disease conditions.

14.11 CONCLUSIONS

Overall, global initiative comprising different sustainable approaches and traditional food systems and food diversity is necessary to prevent and manage the present and future epidemic of metabolic syndrome associated type 2 diabetes and cardiovascular diseases. This is also relevant for other major chronic diseases linked to cancer, liver health, cardiopulmonary, and mental health. A healthy diet including specifically selected and designed bioactive-enriched fruits and vegetables would not only help to counter specific diseases but can also provide us with better long-term immunity for disease prevention by maintaining cellular homeostasis in a redox environment that is under constant free radical attack through cumulative imbalances. It is important to understand that fruits and vegetables in whole food form and their bioactive compounds alone cannot cure these diseases, but along with other important biological, pharmaceutical, and lifestyle factors they can play a significant role in disease prevention or in the reduction of the risk of noncommunicable diseases, especially that of type 2 diabetes and cardiovascular disease.

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15 Enhancing Functional Food Ingredients in Fruits and Vegetables

Shaila Wadud and Gopinadhan Paliyath

15.1 INTRODUCTION

The global population, which reached 6 billion in late 1999, has been estimated to climb to 8.3 billion by 2020. Most of this increase will occur in the cities of the developing world (Miflin, 2000). Problems of politics, distribution, and poverty often create food shortages and undernutrition in developing countries (Zimmermann and Hurrell, 2002). The vast majority of the world's population is dependent on plants as a main source of food. Plants contain several mineral nutrients, vitamins, and numerous phytochemicals that have been shown to have potentially beneficial effects on health especially against the initiation or progression of degenerative diseases (Lindsay, 2002). With the exception of vitamins B_{12} and D, almost all human nutrients can be obtained from plant foods. However, not all plant foods contain all the essential nutrients needed for human health. Also, they usually do not contain all the nutrients in sufficiently concentrated amounts to meet daily dietary requirements in a single serving (Grusak and DellaPenna, 1999). Thus, a diverse, complex dietary mix containing the concentration of various nutrients is necessary to fully met nutritional requirements and to support human growth and health (Grusak and DellaPenna, 1999).

In developing countries, many people do not consume a sufficiently diverse diet and many low-income families exist on a simple diet composed primarily of staple foods such as rice, wheat, maize (poor sources of some macronutrients and many micronutrients) (Calloway, 1995). As a result, consumption of cereal staples, roots, or pulses, with little or no meat or dairy products could lead to severe nutritional deficiencies. It has been reported that over 2 billion people, one-third of the world's population, suffer from vitamin A, Zn, and/or Fe deficiencies (FAO, 1997). Again the prevalence of these nutrient deficiencies, and their associated morbidity and mortality, is highest in developing countries. Because poverty limits food access for much of the developing world's population, improved nutritional quality may help solve problems encountered in cases where plant foods are the major or sole source of food (Galili et al., 2002). Therefore, interest in improving the nutritional quality of plant foods, with respect to both nutrient composition and concentration to ensure an adequate dietary intake of all essential nutrients and to increase the

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consumption of various health-promoting compounds, is of major interest (Grusak and DellaPenna, 1999).

15.2 STRATEGIES FOR NUTRITIONAL ENHANCEMENT

There are several approaches which have been used for improving the nutritional quality of plant foods. Among these are (Lindsay, 2002)

- "the application of traditional breeding methods to select for varieties with an increased level of the bioactive compound"
- "the use of genetic manipulation to introduce new traits in plants"
- "improvements in handling, storage and food processing technologies"

Each of these approaches has a role to play, but genetic manipulation provides a mechanism for the improvement of nutritional quality that overcomes the problem of the absence of a specific biochemical pathway in plant. Potential strategies for the enhancement of specific metabolites could include overexpression of enzymes in metabolic pathway, controlling metabolite degradation by silencing the genes in the degradation pathway, potential increase in the organelles where the pathway is localized, for example, chloroplast and so on, through genetic engineering (Fig. 15.1).

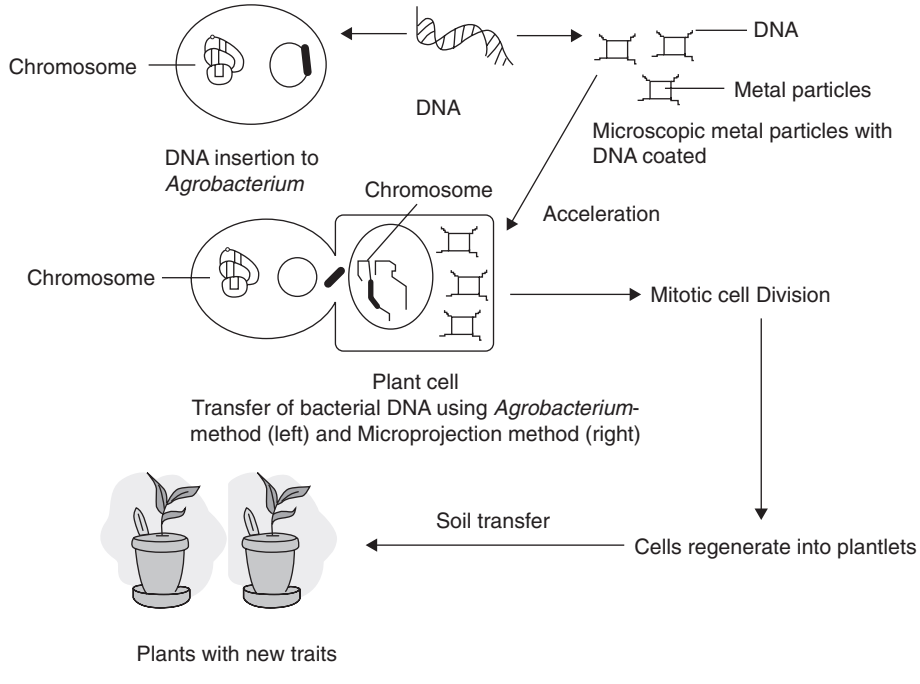


Figure 15.1. *Agrobacterium*-mediated method and microprojection method in order to have genetically engineered plant.

15.3 IMPROVING THE MINERAL CONTENT OF PLANT FOODS

Minerals can be grouped into several categories as mentioned below (Kabata-Pendias and Pendias, 1992):

1. the macronutrient minerals (N, S, P, Ca, K, Mg) that are needed in highest concentration by plants (milligram/gram dry weight range)
2. the micronutrient minerals (Fe, Mn, B, Cl, Zn, Cu, Mo, Ni) that are needed in lesser amounts (microgram/gram dry weight range),
3. other essential minerals (e.g., Na, F, Se, Cr, I) that are found in plant tissues in varying concentrations.

From any one of these categories, a plant's ability to increase the total content of a mineral always depends on soil composition and the availability of that mineral in the plant's environment.

In order to develop strategies for improving the content of any given mineral, a holistic understanding of the relevant transport and partitioning mechanisms, and the molecular to whole-plant factors that regulate them, is very important (Grusak and DellaPenna, 1999). As can be seen from Figure 15.2, the mineral composition of each plant organ is determined by a sequence of events that begins with membrane transport into the roots and aqueous

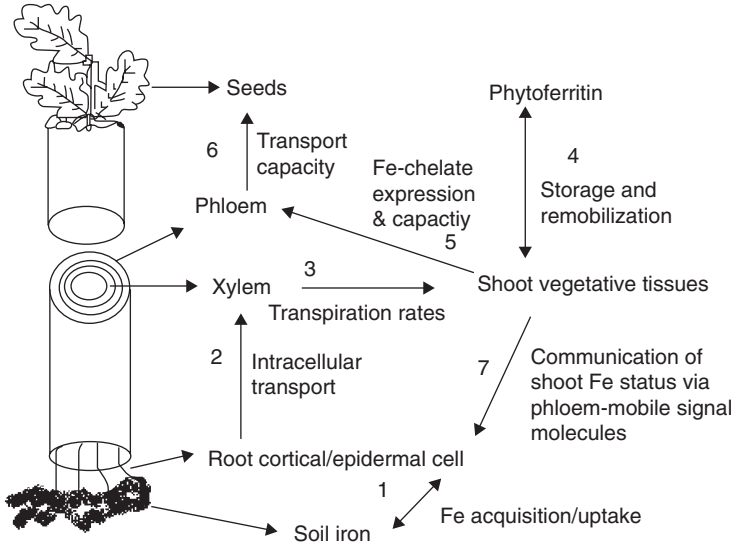


Figure 15.2. Schematic diagram showing Fe transport and accumulation in higher plant tissues. Potential control points that would influence the movement of Fe from one compartment to the next include Fe acquisition/uptake phenomena, including the release of compounds by roots to chelate or to solubilize soil Fe; intracellular/intercellular transport, including the involvement of xylem parenchyma; transpiration rates of vegetative tissues; storage and remobilization phenomena; Fe-chelate expression and capacity for phloem Fe loading; phloem transport capacity of photoassimilates from a given source region; communication of shoot Fe status via phloem-mobile signal molecules to regulate root processes (adapted from Grusak and DellaPenna, 1999).

transport into the xylem system for transit to the vegetative organs. The transport processes may also involve phloem translocation and deposition into cellular compartments for temporary storage in stem or leaf tissues (Grusak and DellaPenna, 1999).

15.3.1 Iron and zinc

Deficiency of iron and zinc are widely prevalent in developing countries. It has been estimated that 40–45% of school-age children are anemic, and approximately 50% of this anemia results from Fe deficiency (WHO/CDC 2004; de Benoist et al., 2007). Groups most affected are all, but especially women and children. Consequences of these deficiencies are reduced cognitive ability, illness from infectious diseases, childbirth complications, and reduced physical capacity and productivity (WHO/CDC 2004; de Benoist et al., 2007). The most common plant sources of iron are green leafy vegetables, legumes, soy foods, nuts, whole-grain and fortified breads, cereals, and pasta, and common sources of zinc are cereals, nuts, legumes, and soy products.

There are several genetic engineering approaches that could be used to increase the Fe content and bioavailability in plant foods. Increasing leaf Fe by 50% (Samuelsen et al., 1998) was reported on a transgenic tobacco plant by expressing a yeast ferric reductase gene, and similar approaches could be used for edible leaf crops. Goto et al. (1999) reported a twofold to threefold increase in Fe in transgenic rice expressing the soybean ferritin gene.

Phytic acid is a major etiologic factor (an absorption inhibitor) for the low bioavailability of Fe and Zn from diets based on staple cereals and legumes (Hurrell, 2002). Minerals, when bound to InsP6 or phytic acid, are hardly or not absorbed in the intestine and are largely excreted, resulting in iron and zinc deficiencies. Phytic acid is known as phytate when in salt form (Fig. 15.3). The manipulation of phytase activity is an important approach to enhancing mineral bioavailability. In rice, the bioavailability of iron has been targeted

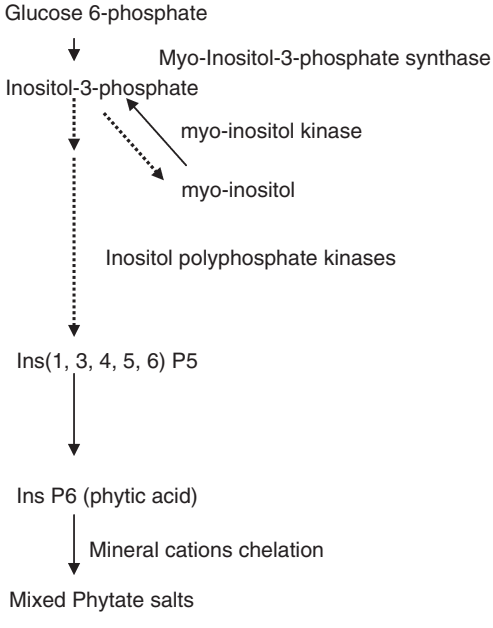


Figure 15.3. Biosynthetic pathway of Phytic acid in plants.

by introducing a gene for a phytase which resulted in a 130-fold increase in the expression of this enzyme (Lucca et al., 2002). The expression of phytase in crops enhances the breakdown of phytic acid and releases minerals.

Breeding strategies have been used to develop low-phytate varieties of maize, barley, rice, and soybean. It has been reported that the Fe bioavailability was 49% greater from tortillas made with low phytic acid maize compared with wild-type maize (Fe absorption 8.2% vs. 5.5%) (Mendoza et al., 1998). Similarly, mean Zn absorption from polenta prepared with the low-phytic acid maize was 78% greater than for wild-type maize (Adams et al., 2000).

15.4 IMPROVING THE ANTIOXIDANTS CONTENT OF PLANT FOODS

Fruits and vegetables contain a wide range of antioxidants including carotenoids such as lycopene and β-carotene, vitamins C and E, and polyphenolics such as quercetin. The consumption of fruit and vegetables has been shown to increase plasma antioxidant levels in human subjects, and this has been associated with reduced blood pressure, which in turn could be a preventative factor in CVD (John et al., 2002). In addition, as described in previous chapters, these compounds not only exert antioxidant activity, but also the signal transduction processes and gene expression, showing preventive effects on other chronic degenerative diseases such as cancer, inflammation, and degenerative disorders.

15.4.1 Lycopene and β-carotene

Lycopene is a carotenoid (an acyclic isomer of β-carotene) which is synthesized by plants and microorganisms but not by animals. It undergoes *cis-trans* isomerization induced by light, thermal energy, and chemical reactions (Zechmeister et al., 1941; Nguyen and Schwartz, 1999). Lycopene from natural plant sources exists predominantly in an all-*trans* configuration, the most thermodynamically stable form. In human plasma, lycopene is present as an isomeric mixture, with 50% as *cis* isomers (Zechmeister et al., 1941). Lycopene is one of the most potent antioxidants, with a singlet-oxygen-quenching ability twice as high as that of β-carotene and 10 times higher than that of α-tocopherol (DiMascio et al., 1989).

Lycopene is a fat-soluble compound and as such is poorly absorbed by the body from fresh fruit. Absorption is much greater from processed tomato products, such as pastes, especially if this involves mixing with fats. Red fruits and vegetables, including tomatoes, watermelons, pink grapefruits, apricots, and pink guavas, contain lycopene. Processed tomato products, such as juice, ketchup, paste, sauce, and soup, are all good dietary sources of lycopene. The release of lycopene from the food matrix due to processing, the presence of dietary lipids and heat-induced isomerization from an all-*trans* to a *cis* conformation enhance lycopene bioavailability. Dietary lycopene may increase the lycopene status in the body and, acting as an antioxidant, may trap reactive oxygen species, increase the overall antioxidant potential, or reduce the oxidative damage to lipid (lipoproteins, membrane lipids), proteins (important enzymes), and DNA (genetic material), thereby lowering oxidative stress. This reduced oxidative stress may lead to reduced risk for cancer and cardiovascular disease (Agarwal and Rao, 2000).

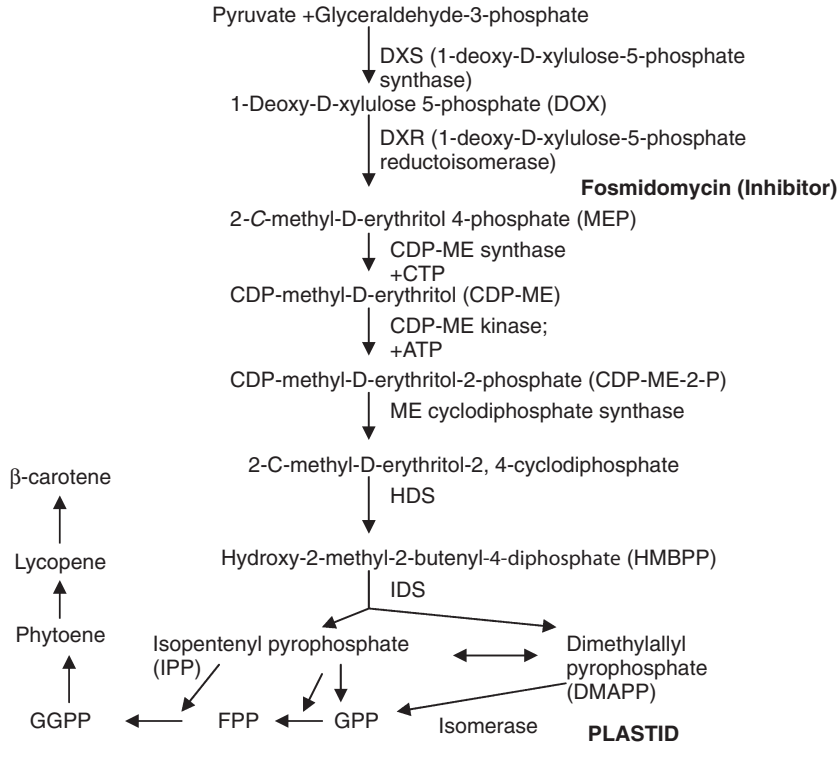


Figure 15.4. The carotenoid biosynthetic pathway in plastid (MEP pathway).

Beta-carotene is also known as provitamin A, because it is one of the most important precursors of vitamin A in the human diet. Vitamin A has several functions in the body. The most well-known is its role in vision. Over 100 million children worldwide are vitamin A deficient, and improving the vitamin A content of their food could prevent as many as two million deaths annually in young children (FAO, 1997). Sources of β-carotene include carrots, yellow and orange fruits such as mangoes, papayas, and yams, and in green leafy vegetables such as spinach, kale, sweet potato leaves, and sweet gourd leaves. Important factors that improve the bioavailability of β-carotene include, cooking, chopping, and the presence of dietary fat.

Carotenoid biosynthesis is achieved through a pathway that has been identified by Schwender et al. (1996), which is localized in the plastids (Fig. 15.4). In this pathway, glyceraldehyde 3-phosphate (GAP) and pyruvate are the first precursors. Pyruvate is first decarboxylated and then coupled to the carbonyl group of GAP yielding 1-deoxy-d-xylulose 5-phosphate and finally isopentenyl pyrophosphate (IPP). The sequential condensation of IPP with dimethylallyl pyrophosphate, an isomer of IPP, results in the formation of the C20 intermediate geranylgeranyl pyrophosphate (GGPP). Head-to-tail condensation of two molecules of GGPP results in the formation of the first C40 carotenoid phytoene by the activity of phytoene synthase. The noncolored phytoene undergoes a series of sequential desaturation reactions to produce phytofluene, ζ-carotene, neurosporene, and finally, the colored lycopene. Finally, cyclization of the two end-groups results in β-carotene formation.

Expression of a gene for phytoene synthase from the bacteria *Erwinia uredovora* in tomato plants resulted in a twofold to fourfold increase in total carotenoid levels and an enrichment of 1.8-fold and 2.2-fold for lycopene and β-carotene, respectively, in the fruit (Fraser et al., 2002). Rice, which is a major world food source, contains poor levels of β-carotene in the endosperm, which is the major tissue consumed as food after mechanical processing of the grain. A β-carotene-enriched "Golden rice" was produced through biotechnology to biosynthesize β-carotene in the edible parts of rice (Ye et al., 2000). Several genes in the carotenoid pathway were introduced into the rice genome through *Agrobacterium*-mediated transfer (Ye et al., 2000), resulting in the expression of the β-carotene biosynthetic pathway in the rice endosperm. The genes for phytoene synthase, phytoene desaturase, and lycopene β-cyclase were obtained from daffodil and the gene for carotene desaturase was of bacterial origin. The β-carotene was expressed in the grain at 1.6–2.0 μg/g fresh weight. Assuming that 6 μg of β-carotene is converted to 1 μg retinol equivalents (REs), 1.2 kg of "golden" rice per day would be necessary to provide all the recommended vitamin A intake of a 4- to 8-year-old child (400 μg RE) (NRC, 1989).

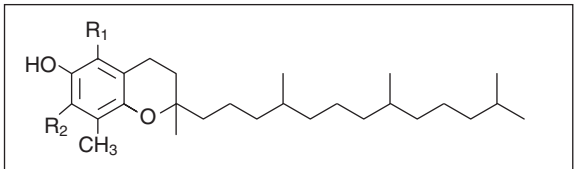
15.4.2 Vitamin E

Tocopherol, or vitamin E, is another important dietary antioxidant that occurs in plants in several isomeric forms (α-, β-, γ-, and δ) with α-tocopherol having the highest vitamin E activity. Plant oils (such as soybean, canola, corn, palm oils) are the main dietary source of tocopherols, and typically contain α-tocopherol as a minor component and higher levels of γ-tocopherol (Shintani and DellaPenna, 1998). Figure 15.5 shows the tocopherol biosynthetic pathway.

U.S. recommended daily allowance (RDA) of vitamin E is in the range of 10–13.4 international units (IU), (equal to 7–9 mg of α-tocopherol) to be consumed daily (NRC, 1989). However, daily intake of vitamin E in excess of the RDA (100–1000 IU) was found to be associated with decreased risk of cardiovascular disease and some cancers, improved immune function, and slowing of the progression of a number of degenerative human conditions (Traber and Sies, 1996). Although the most highly consumed vegetable oils in diets (i.e., soybean, corn, and rapeseed oils) (American Soybean Association, 1997) contain high levels of total tocopherol, these oils are relatively poor sources of α-tocopherol (the form with the highest vitamin E activity), while containing a higher level of γ-tocopherol. γ-Tocopherol is methylated to form α-tocopherol in a reaction catalyzed by the enzyme γ-tocopherol methyltransferase (γ-TMT). These observations suggest that γ-TMT activity is likely limiting in the seeds of most agriculturally important oil crops and may be responsible for the low proportion of α-tocopherol synthesized and accumulated. That is why γ-TMT is a prime molecular target for manipulation of α-tocopherol levels in crops. Overexpression of γ-tocopherol methyltransferase in *Arabidopsis* seeds shifted oil compositions in favor of α-tocopherol. This may suggest that overexpressing of the γ-TMT gene in crops such as soybean, canola, corn, and palm oils should similarly elevate α-tocopherol levels and thereby increase the nutritional value of these important dietary sources of vitamin E (Shintani and DellaPenna, 1998).

15.4.3 Flavonoids

Flavonoids are a group of polyphenolic plant secondary metabolites important for plant biology and human nutrition. They can be grouped into different classes based on their



Type	R_1	R_2
α-tocopherol	CH_3	CH_3
γ-tocopherol	H	CH_3
β-tocopherol	CH_3	H
δ-tocopherol	H	H

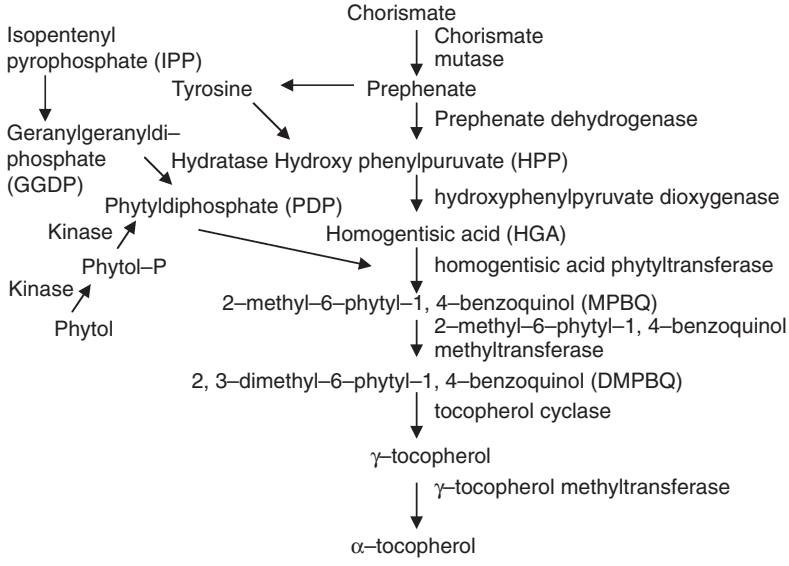


Figure 15.5. Tocopherols and their biosynthetic pathway. The pathway shown is present in all photosynthetic organisms. The sequence of steps to α-tocopherol after addition of the phytyl tail to HGA is the most widely accepted of many possible sequences proposed from biochemical studies. HPPDase is generally accepted as having a cytosolic localization; all other enzymes are presumably localized to plastids (adapted from Grusak and DellaPenna, 1999).

core structure, the aglycone, such as chalcones, flavanones, dihydroflavonols, flavonols, and anthocyanins In particular, flavonols are potent antioxidants, and their dietary intake is correlated with a reduced risk of cardiovascular diseases (Bovy et al., 2002). Figure 15.6 shows the flavonoid biosynthesis pathway. Several fruits, specifically the berry fruits, tender fruits, and the pome fruits and purple vegetables are enriched in several types of polyphenols including anthocyanins. Tomato fruits are not very rich in phenolic components or anthocyanins. Even though some genotypes may be enriched in phenolic components, these are not widely cultivated. In the normal fruit, flavonoids are at a low level and are localized in the skin. The induction of flavonoid synthesis in the flesh of the tomato fruit was achieved by expression of two transcription factors, genes *LC* and *C1*, from maize.

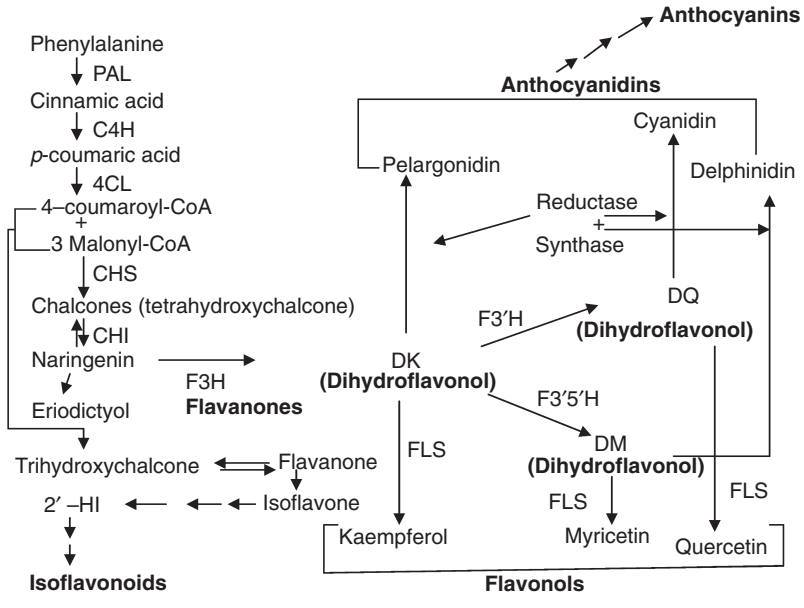


Figure 15.6. Scheme of the flavonoid biosynthesis pathway (adapted from Bovy et al., 2002). PAL: Phe-ammonia lyase; C4H: cinnamic acid 4-hydroxylase; 4CL: 4 coumarate : coenzyme A ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone-3-hydroxylase; 2′HI: hydroxy isoflavanone; FLS: flavonol synthase; F3′H: flavanone-3-hydroxylase; F3′5′H: flavanone-3′5′-hydroxylase; DK: dihydrokaempferol; DM: dihydromyricetin; DQ: dihydroquercetin.

It was found that expression of both genes was required and sufficient to upregulate the flavonoid pathway in tomato fruit flesh, a tissue that normally does not produce any flavonoids. These fruits were found to accumulate high levels of the flavonol kaempferol and, to a lesser extent, the flavanone naringenin in their flesh (Bovy et al., 2002). Expression of two transcription factors from snapdragon in tomato fruits resulted in the induction of anthocyanin biosynthesis, making the fruits intensely purple colored in both the flesh and the skin. Feeding purple tomatoes to cancer-susceptible mice resulted in an enhancement of their life span (Butelli et al., 2008).

15.5 IMPROVING THE AMINO ACID CONTENT OF PROTEINS OF PLANT FOODS

The nutritive value of a protein-based diet is directly related to the content of these essential amino acids. In general, cereals have proteins with a low content of the essential amino acids lysine, threonine, and tryptophan, while legume proteins have a deficiency of cysteine, methionine, and tryptophan. Figure 15.7 shows the biochemical regulatory mechanism proposed to regulate the synthesis of the amino acid lysine, in higher plants. It can be seen that lysine synthesis is finely regulated by a feedback mechanism; that is, when the lysine content is high, there is an inhibition of two of the enzymes involved in the metabolic pathway leading to the synthesis of this amino acid. The transformation of soybean with the gene *lysCM4* from *Escherichia coli* (encoding AK) and the *dapA* gene from

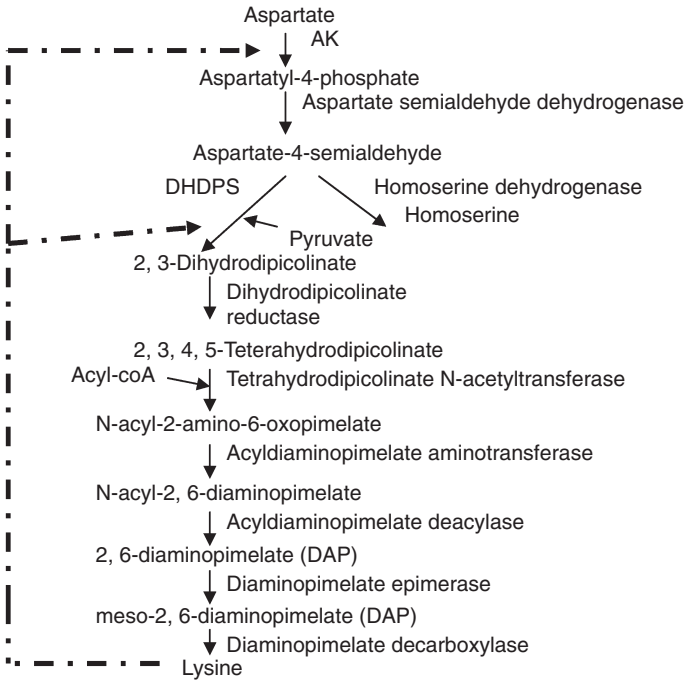


Figure 15.7. Regulatory mechanism proposed to regulate the synthesis of the amino acid lysine, in higher plants, derived from the amino acid aspartate. Enzyme activities, aspartate kinase (AK), and dihidrodipicolinate synthase (DHDPS) are indicated (adapted from Amaya et al., 2002). Broken lines indicate the inhibition of these enzymes by lysine.

Corynebacterium (encoding DHDPS), both insensitive to the feedback inhibition by lysine, resulted in a transgenic soybean plant. The lysine content was found to be over 100-fold the value of the untransformed plants (Amaya et al., 2002).

15.6 IMPROVING THE FATTY ACID COMPOSITION OF PLANT SEED OIL

Lipids are also main components of the human diet. The consumer preference for plant-derived oils is increasing. Common fatty acids in the commercial seed oils are lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic. Figure 15.8 shows the interconversion of fatty acids.

It has been shown in the epidemiological studies that intake of monounsaturated acids was associated with a low incidence of coronary artery disease (Keys et al., 1986). This has been explained by its reduction in the low-density lipoproteins (LDL) levels and their oxidation (Mata et al., 1997). Therefore, several studies have been targeted to the modification of fatty acids by increasing the level of unsaturation. As for example, oleic acid, the major monounsaturated acid of the diet, reduces cholesterol and LDL in the serum. Overexpression of the desaturase gene in soybean that encodes for the enzyme catalyzing the conversion of the saturated precursor stearic acid into oleic acid resulted in the eleva-

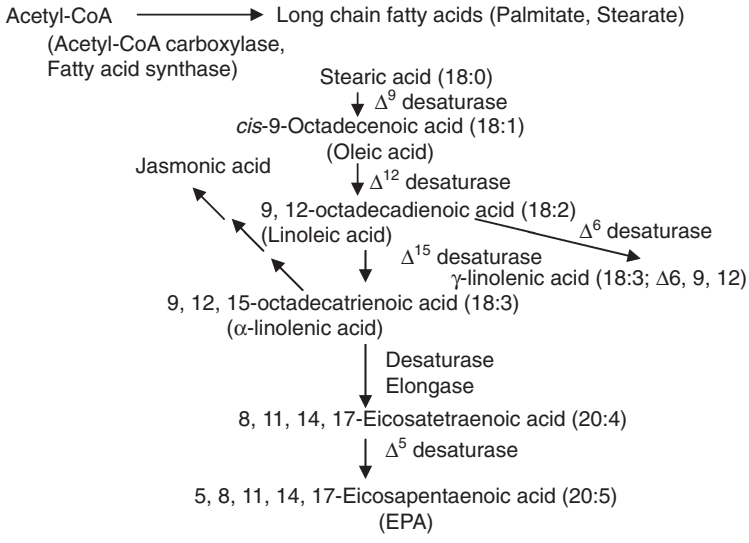


Figure 15.8. Interconversions of the fatty acids. Enzymes: Δ^nDS: n-desaturase: EL: elongase (adapted from Amaya et al., 2002).

tion of oleic acid content of soybean up to 80% of the total fatty acids content of the seeds (Kinney, 1996).

15.7 INFLUENCE OF PROCESSING AND STORAGE IN THE NUTRITIVE VALUE OF PLANT FOODS

15.7.1 Processing of plant oils

There are several components in vegetable oils which have been shown to have health beneficial effects. One of the important nutritional characteristics of the plant oil is the fatty acid composition. In addition, the plant sterols, carotenoids, and the tocopherols and a range of plant polyphenols characterized from plant oil were shown to have important health-promoting properties. Refining of crude vegetable oils usually are carried out by using water or steam to de-gum, followed by physical (steam treatment, bleaching) or chemical (saponifcation with sodium hydroxide, centrifugation) methods. These refining procedures remove carotenoids and some other antioxidants. To improve overall product quality and avoid rancidity, it is very critical to improve these technologies to retain high levels of antioxidants throughout the processing stages (Lindsay, 2000).

During olive oil extraction, a two-phase technology has shown improvement in the oil quality when compared with those that use the conventional three-phase equipment. A higher content of polyphenols, ortho-diphenols, and tocopherols, as well as a higher stability, was reported while using the two-phase technology (Lindley, 1998).

15.7.2 Processing of fruits and vegetables

Optimal processes for maintaining the content of antioxidant vitamins in fruit and vegetables start from postharvest handling. Careful attention should be given to temperature in

harvesting. Minimization of light and oxygen helps reduce losses. Appropriate humidity helps to preserve vitamin content during the cold storage of vegetables. Again, the vitamin content of fresh fruits is not stable for long periods of time in the refrigerator. By inactivating polyphenol oxidase, it is possible to maintain antioxidant status (Lindley, 1998).

In fruit and vegetable marketing, modified atmosphere packaging is widely used. The removal of oxygen, and the use of plastics with low gas permeability membranes help to stabilize the nutrient content. However, carbon dioxide accumulation occur, leading to anoxia and loss of quality. These problems could be overcome by using active packaging which uses oxygen scavenging materials such as vitamins C or E into the film (Lindley, 1998, Shetty et al., 2008).

Processing can also affect the chemical composition of the food that may have biological consequences such as significant losses of some carotenoids because of *trans* to *cis* isomerization (e.g., 5-*cis* lycopene and 13-*cis*-β-carotene), as well as to different carotenoid by-products. The extent of changes is dependant upon the type of vegetable, the method of cooking, and the temperature and time conditions. Higher temperatures and prolonged period of cooking result in deteriorative changes (Nguyen and Schwartz, 1998). New processing techniques, such as high electric pulse fields, or high pressure processing especially of fruit juices, have been shown to maintain vitamin levels.

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