FUNCTIONAL FOODS, NUTRACEUTICALS AND NATURAL PRODUCTS

CONCEPTS AND APPLICATIONS

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Preface

The demand for foods with a positive impact on human health and wellness has exploded globally over the past two decades. This growth is driven by socioeconomic and scientific factors, including increases in population, disposable income, life expectancy and healthcare costs. The market for healthier foods is also enhanced by advancements in our understanding of dietary bioactive ingredients and their effects on various aspects of human health at a systems and molecular level. This book examines the rapidly growing field of functional foods in the prevention and management of chronic and infectious diseases. It attempts to provide a unified and systematic account of functional foods by illustrating the connections among the different disciplines needed to understand foods and nutrients, mainly: food science, nutrition, pharmacology, toxicology and manufacturing technology. Advances within and among all these fields are critical for the successful development and application of functional foods. Chapters in the present volume explore the varied sources, biochemical properties, metabolism, health benefits and safety of bioactive ingredients. Special emphasis is given to linking the molecular and chemical structures of biologically active components in foods to their nutritional and pharmacological effects on human health and wellness. In addition to discussing scientific and clinical rationales for different sources of functional foods, the book also explains in detail scientific methodologies used to investigate the functionality, effectiveness and safety of bioactive ingredients in food. This text is intended for food, nutrition, medical specialists as well as students, and will give the

reader a systematic and in-depth understanding of basic and advanced concepts in functional foods.

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Functional Foods—History and Concepts

S. ARAI, D.A. VATTEM and H. KUMAGAI

1.1. BACKGROUND

The concept originating in ancient China and transported to Japan long ago—"Medicine and food are isogonics" (Arai 2005)—as well as the doctrine of Hippocrates (460–377 B.C.), "Let food be thy medicine and medicine be thy food," (Hasler 2001)—has had a resurgence. The advent of up-to-date science and sophisticated technology has made it possible to recognize food as supplying us with more than nutrition. Food can even help reduce the risk of chronic lifestyle-related diseases such as diabetes, dyslipidemia, hypertension, obesity, etc., caused by inadequate metabolic modulation, and cancer, allergies, infection diseases, etc., caused by broken body-protection systems. The recent trend of reconsidering foods and their proper intakes as the first line of defense against these abnormal modalities has grown from our increased understanding of physiological rather than nutritional benefits of foods. Against this backdrop, the terminology and concept of "functional food" were born in Japan about two dozen years ago (Arai 2005).

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In 1984, a large-scale national project to be carried out by an ad hoc research team headed by Professor S. Arai at the University of Tokyo and other leading food scientists was launched under the egis of the Japan Ministry of Education, Science, and Culture. The project was headquartered at the University of Tokyo which has studied "food for health" since 1910 when the outstanding biochemist Dr. Umetaro Suzuki investigated rice bran and found an antiberiberi principle oryzanin (Suzuki *et al.* 1912) which was then renamed thiamin (vitamin B1). With it as a background, the project had humble beginnings to trace a unique path of development through special insights into physiologically functional non-nutrients originating in common foods (see Table 1.1). The research team first proposed three categories of food functionality (see Figure 1.1) and then pinpointed its study on the "tertiary" function to be evoked by non-nutritive components. Political actions for new legislation followed. In 1991, the Japan Ministry of Health and Welfare initiated the world-first policy of legally permitting the commercialization of selected functional foods named "food for specified health use" (FOSHU). Each of the FOSHU products can claim a certain degree of health benefit. These scientific and political activities were reported in *Nature* news (Swinbanks and O'Brien 1993) with the headline, "Japan explores the boundary between food and medicine;" this reminded us of the old day's saying, "Medicine and food are isogonic."

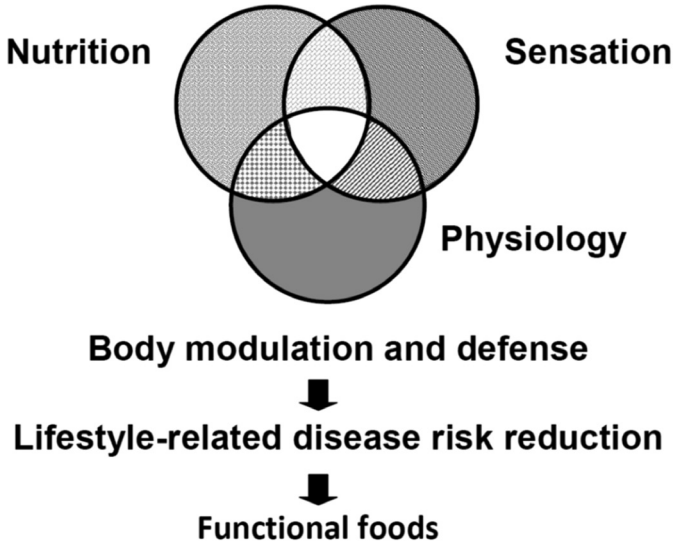

FIGURE 1.1. *Three categories of food functions proposed.*

TABLE 1.1. Common Non-nutritive Components with Possible Functions for Disease Risk Reduction.

Component	Example (origin)	Disease
Carotenoid		
Carotene	Lycopene (tomato)	Oxidative stress
Xanthophyll	Lutein (vegetable)	Oxidative stress
Flavonoid		
Flavonol	Quercetin (vegetable)	Oxidative stress
Flavone	Nobiletin (citrus fruit)	Oxidative stress
Isoflavone	Daidzein (soybean)	Osteoporosis
Flavanone	Naringin (grapefruit)	Diabetes
Catechin	Epigallocatechin gallate 3- or 4-methyl ester (tea)	Allergy
Anthocyanin	Cyanidin (kidney bean)	Oxidative stress
Simple polyphenol	Chlorogenic acid (coffee)	Cancer
Phenyl propanoid	Curcumin (turmeric)	Cancer
Isoprenoid	Ubiquinone (ubiquitous)	Oxidative stress
Triterpenoid	Soyasaponin (soybean)	Oxidative stress
Phytosterol	β-Sitosterol (soybean)	Hypercholesterol-emia
Chromanol derivative	Tocotrienol (soybean)	Oxidative stress
Isothiocyanate	Sulforaphane (broccoli)	Detoxification
Sulfoxide	Alliin (garlic)	Coagulation
Vanilloid	Capsiate (chili)	Obesity
Alkaloid	Caffeine (coffee)	Obesity
Lignan	Sesamin (sesame)	Hangover
Organic acid	Acetic acid (vinegar)	Hypertension
Amino acid	γ-Aminobutyric acid (rice)	Hypertension
Protein	β-Conglycinin (soybean)	Obesity
Oligopeptide	Val-Pro-Pro (sour milk)	Hypertension
Lipid	3-Diacylglycerol (cooking oil)	Obesity
Polysaccharide	Alginic acid (seaweeds)	Hypercholesteremia
Prebiotics	Manno-oligosaccharide (coffee)	Obesity
Probiotics	*Bifidobacterium lactis* (yogurt)	Constipation
Sweetening	Neoculin (*Curculigo latifelia*)	Diabetes

1.2. SCIENTIFIC PERSPECTIVES

What may be most important at present is the evaluation of the function of a food. Since any functional food must be based on scientific principles, its evaluation should essentially depend on data from biochemistry, physiology, molecular biology, and most other modern bio-

sciences. A key approach to the development of a functional food is the identification and validation of relevant markers, including biomarkers, which can predict its potential benefits relating to a target function in the body. If a marker represents an event directly involved in the process, it should be considered as a functional "factor." However, if a marker represents a correlated event, it should be considered as a function "indicator" (Bellisle *et al.* 1998; Anonymous 2002).

A similar but even lager national project, headed by Professor Arai, started with the title "Non-nutritive functional components in foods: analysis and systematization." It emphasized the significance of trooping a variety of methodologies, with research targets hierarchized as in Figure 1.2 (Arai 2006). The project was successfully undertaken, with its administration operated by Professor M. Uehara at Tokyo University of Agriculture. Scientifically, the research teams investigated hundreds of fruits and vegetables for functional food components and in particular, Professor K. Kanazawa at Kobe University, presented an interesting polyphenol pyramid (see Figure 1.3) (unpublished).

Meanwhile, the genome programs on humans and other representative organisms for scientific and industrial uses were almost completed, enabling us to obtain the genome information by internet services. The

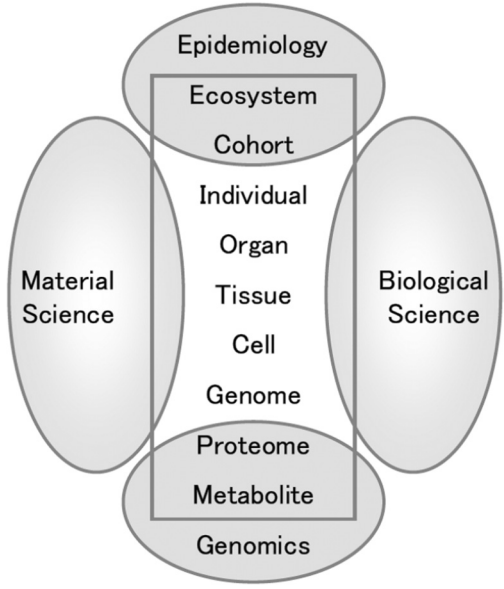

FIGURE 1.2. *Systematized methodologies for analysis of the "tertiary" functions of food and hierarchal targets of analysis.*

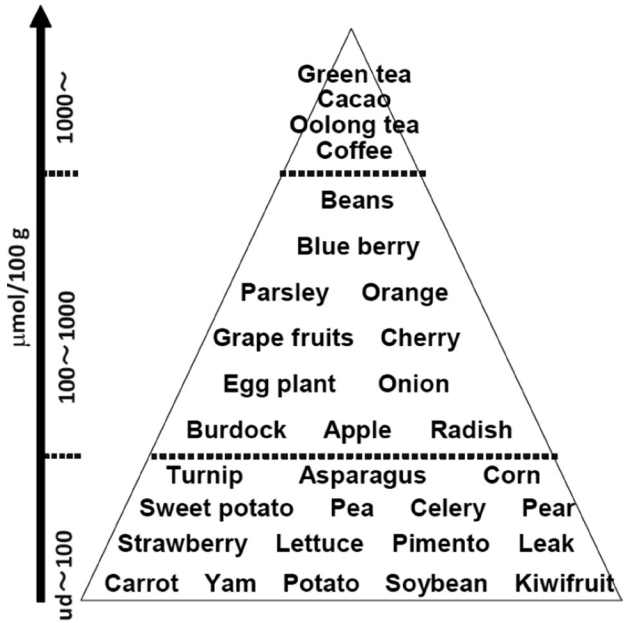

FIGURE 1.3. *Polyphenol pyramid.*

use of the DNA microarray technique has made it possible to qualitatively and quantitatively investigate statistically significant changes in gene expression that took place at target tissues of the body after ingesting individual foods or their components. A new science, coined as "nutrigenomics," has thus come into being (Maller and Kersten 2003); the term "functional food genomics" is sometimes used in the particular case that the physiological functionality of a whole food is emphasized more than its nutrition.

In the meantime, Professor K. Abe at the University of Tokyo organized the International Life Science Institute (ILSI) Japan-endowed chair "Functional Food Genomics" (with Professor Y. Nakai in charge) which aims to explore a new dimension of food science in the form of academia-industry consortium (Nakai *et al.* 2010). This laboratory has publicized DNA microarray data on soy protein isolate (Tachibana *et al.* 2005), cocoa as a whole (Matsui *et al.* 2005), sesamin (Tsuruoka *et al.* 2005), apple polyphenol (Ohta *et al.* 2006), grape seed (Sano *et al.* 2007), royal jelly as a whole (Narita *et al.* 2006), fructo-oligosaccharide (Fukasawa *et al.* 2007), vinegar as a whole (Nakano *et al.* 2004), tomato (Aizawa *et al.* 2009), and others. What may be extremely interesting would be the data on inhaled linalool as an aroma that reduces a kind of

TABLE 1.2. Foods for Specified Health Uses, with Their Numbers Bracketed in Italic Type.

Category 1	Modulation of gastrointestinal conditions 1. Oligosaccharides: lactosucrose *[28]*; galacto-oligosaccharides *[14]*; coffee bean manno-oligosaccharides including mannobiose *[14]*; fructo-oligosaccharides *[7]*; soybean oligosaccharides *[6]*; xylo-oligosaccharides *[4]*; isomaito-oligosaccharides *[4]*; lactulose *(Hassler et al. 2004)*; raffinose *(Hassler et al. 2004)* 2. Lactic acid bacteria: *Lactobacillus casei* Shirota *[30]*; *L. acidophilus* CK92 and *L. helveticus* CK60 *[7]*; *Bifidobacterium lactis* Bb-12 *[7]*; *L. delbrueckii* subsp. *bulgaricus* 2038 and *Streptococcus salivarius* subsp. *thermophilus* 1131*[6]*; *B. breve* Yakult *[6]*; *B. longum* BB536 *[6]*; *L. casei* SP and *B.* SP *(Hassler 2002)*; *B. lactis* LKM512 *(Hassler 2002)*; *B. lactis* FK120 *(Hassler 2002)*; *L. casei* NY1301 *(Hassler 2002)*; *L.* GG *(Hassler 2002)*; *L. casei* LC1 *(Hassler 2002)* 3. Dietary fibers: indigestible dextran *[141]*; psyllium husk *[22]*; wheat bran *[4]*; gum guaic hydrolysate *[6]*; agar *(Katan and De Roos 2002)*; polydextrose *(Hassler 2002)*; low-molecular-weight sodium alginate *(Hassler 2002)*; low-molecular-weight sodium alginate and water-soluble corn fiber *(Hassler et al. 2004)*; indigestible dextran and wheat bran *(Hassler et al. 2004)*; dietary fiber from beer yeast *(Hassler et al. 2004)*; indigestible dextran, reduced type *(Hassler et al. 2004)*; indigestible starch *(Hassler et al. 2004)* 4. Other components: milk whey fermented by propionic acid bacterium *(Katan and De Roos 2002)*; *Bacillus subtilis* K-2 *(Hassler et al. 2004)* 5. Combination of chemically defined components: galacto-oligosaccharide-polydextrose *(Hassler et al. 2004)*
Category 2	Modulation of serum cholesterol level Chitosan *[47]*; soybean protein *[27]*; phospholipid-binding soybean peptides including Cys-Ser-Pro-His-Pro *[19]*; low-molecular-weight sodium alginate *[6]*; phytosterol *[5]*; phytosterol ester *(Katan and De Roos 2002)*; broccoli-cabbage peptide Ser-Mer-Cys-Ser *(Hassler 2002)*; tea catechin *[4]*
Category 3	Modulation of serum cholesterol level and gastrointestinal conditions Dietary fiber from psyllium husks *[19]*; low-molecular-weight sodium alginate *[9]*
Category 4	Modulation of blood pressure Sardine peptide including Val-Tyr *[65]*; lactotripeptides Val-Pro-Pro and Ile-Pro-Pro *[12]*; dried bonito “katsuobushi” oligopeptides *[7]*; “wakame” seaweed peptides including Pro-Tyr-Val-Tyr and Ile-Tyr *[4]*; γ-aminobutyric acid *[9]*; casein dedocapeptide *(Hassler et al. 2004)*; Ile-Tyr *[4]*; acetic acid *[5]*; sesame peptides including Leu-Val-Tyr *(Hassler 2002)*; “nori” seaweed oligopeptides including Ala-Lye-Tyr-Ser-Tyr *(Hassler 2002)*; “tochu” loaf glycoside *[4]*; royal jelly peptide including Val-Tyr, Ile-Tyr and Ile-Val-Tyr *(Hassler 2002)*; yanlong tea flavonoids including hyperoside and isoquercitrin *(Hassler et al. 2004)*; chlorogenic acid *(Hassler 2002)*

(continued)

TABLE 1.2 (continued). Foods for Specified Health Uses, with Their Numbers Bracketed in Italic Type.

Category 5	Acceleration of mineral absorption Casein phosphopeptides *(Katan and De Roos 2002)*; heme iron *(Katan and De Roos 2002)*; calcium citrate-malate *(Hassler et al. 2004)*
Category 6	Acceleration of mineral absorption and modulation of gastrointestinal conditions Lactosucrose *(Hassler 2002)*; fructo-oligosaccharide *(Hassler et al. 2004)*
Category 7	Promotion of bone health Soybean isoflavones *[13]*; vitamin K as menaquinone-7 *[7]*; fructo-oligosaccharides *[5]*; polyglutamic acid *(Hassler et al. 2004)*; milk basic protein *(Hassler et al. 2004)*; and calcium *[13]* vitamin K-2 as menaquinone-4 *(Hassler 2002)*
Category 8	Maintenance of healthy teeth Combined xylitrol-maltitol-calcium monohydrogen phosphate-"fukuronori" seaweed furanone *[23]*; maltitol *(Hassler 2002)*; green tea fluorine *[7]*; combined xylitol-calcium monohydrogen phosphate-"fukuronori" seaweed flavanone *(Hassler et al. 2004)*; combined maltitol-palatinose-green tea polyphenols *(Hassler et al. 2004)*; combined maltitol-hydrogenated palatinose-erythritol-green tea polyphenols *(Hassler et al. 2004)*; soybean isoflavone-calcium *(Hassler et al. 2004)*; Combined palatinose-green tea palatinose polyphenols *(Hassler et al. 2004)*; combined xylitol-reduced palatinose calcium monohydrogen (Katan and De Roos 2002); milk protein hydrolysate containing casein phohphopeptide *[27]*; phosphorylated oligosaccharide calcium *[6]*
Category 9	Modulation of blood sugar level Indigestible dextrin *[136]*; wheat albumin *[5]*; bean extract *(Hassler 2002)*; arabinose *(Hassler et al. 2004)*; guava leaf polyphenol *(Hassler et al. 2004)*; resistant recrystallized amylose *(Hassler et al. 2004)*
Category 10	Modulation of serum triacylglycerol level and blood fat percentage Globin hydrolysate containing Val-Val-Tyr-Pro *[14]*; tea catechin *[15]*; middle-chain fatty acid *[5]*; coffee bean manno-oligosaccharides including mannobiose *[20]*; oolong tea polymerized polyphenol including oolong homo-bis-flavan B *(Hassler 2002)*; fermented soy bean extract *(Hassler et al. 2004)*; β-conglycinin *[5]*; EPA and DHA *[4]*

restrained stress (Nakamura *et al.* 2009), and those on a kind of isolation stress that induces dyslipidemia (Motoyama *et al.* 2009).

The use of genomics is already an international trend. Nutrigenomic research institutions around the world are forming consortia to consolidate their activities aiming at evidence-based functional food science through cooperation in the development of systems biology. In North America, the National Institutes of Health (NIH) provides financial sup-

port for an ongoing research project that is focused on the relationship between diet and gene and between diet and disease in collaboration with nine research centers and four external affiliated organizations. Europe witnessed a launch of a large-scale, 6 year project in January 2004 that was kicked off with the participation of 22 research institutions from ten countries. The European Union earmarked a budget of 17.3 million Euro for this project. New Zealand and Australia play a leading role in Oceania to set up a research organization that specializes in gut health research. These initiatives drew in Canada, China, South Korea, Singapore, and other countries to form a global network. These countries have each launched a clear strategy to exchange information with each other and to invest their research resources in the domains where they can capitalize on their own strengths (Kuwata 2006). Globally, a myriad of papers have been presented so far; some selected reports even within the recent few years are listed as references (Jew *et al.* 2009; Boue *et al.* 2009; Sirtori *et al.* 2009; Eusson *et al.* 2010; Schiepers *et al.* 2009; Berendschot *et al.* 2009; Naito *et al.* 2009; Johnston 2009; Porrini 2008; Medic-Saric *et al.* 2009; Laparra and Sanz 2010; Mullie *et al.* 2009; Minervini *et al.* 2009). The readers are also recommended to consult several volumes (in English) with the term "functional foods" in their book titles (Goldberg 1994; Mazza 1998; Gibson and Williams 2000; Wildman 2001; Hurst 2002; Shi *et al.* 2002; Watson 2003; Korver *et al.* 2004; Eisenbrand 2004; Neeser and German 2004; Hasler 2005).

1.3. POLITICAL STATES

The implementation of the FOSHU policy, as well as the initiation of functional food science in Japan, had a strong impact on many countries in the world, particularly in Europe. In 1995, the UK Ministry of Agriculture, Fisheries, and Food defined (although temporarily) functional foods as those having components incorporated that confer specific medical or physiological benefits, other than nutritional effects (Bellisle *et al.* 1998). Greater interest was shown by ILSI Europe which addressed the present status by claiming that we stand today at the threshold of a new frontier in nutritional science. They also stated that the concept of food is changing from a past emphasis on eating to sate hunger, into an emphasis on the potential uses of food to reduce the risk of chronic illness (Anonymous 2002). This new concept is of extreme importance in view of the demands of the elderly population for an

improved quality of life, increased life expectancy, and reduced costs for health care. A key approach for the science of functional food to develop in compliance with these demands is, among others, the identification and validation of relevant markers which can predict some potential benefits in the body. It is thus of crucial importance to define the health claim for each functional food by the three different phrases: (1) nutrient function claims, (2) other function claims, and (3) reduction of disease risk claims. The former two would be defined based on genomics data as well as biochemical biomarkers (Bellisle *et al.* 1998). Health claims should always be scientifically defined in harmonization with global standards. Talking of the FOSHU system, the goal is to evaluate its physiological functionality for formal approval. On the other hand, the enhanced functional claims and the disease risk reduction claims were proposed by both the Codex and EU project in 1999. The structure/function claims were expressed in the Dietary Supplement Health Education Act in the United States in 1994. The nutrient function claim was included in the guidelines adopted by the Codex in 1997. The generic claims primarily include nutrient function claims based on well-established, generally accepted knowledge and could be standardized without specific, individual substantiation. Innovative claims such as enhanced function claims or structure/ function claims, however, should be evaluated individually by independent experts in order to protect consumers from false or misleading descriptions. For this scientific substantiation, in addition to animal studies and in vitro studies, we also need human intervention studies (Shimizu 2003). The FOSHU system has been making good progress. Up to the present time (June 1, 2010), the Ministry has approved 941 FOSHU products categorized into 10 health claims (see Table 1.2) (Arai *et al.* 2008). Each product is permitted to claim a certain degree of health benefit as long as the claim does not overestimate its efficacy for disease risk reduction. The legal framework is also expected to stop ill-defined, misleading product advertisements. It is added that a close attention has been nationally and internationally paid to the safety of functional foods. In particular, the ILSI Annual Meeting 2010 in Rio Grande has had a scientific session of "Risk Assessment and Functional Foods," which comprises topics on the mode of action of functional foods in human relevance framework, their threshold of toxicological concern, and so on.

As discussed above, the name of "functional food" as well as its concept has been globally accepted enthusiastically, and the science is in progress for further development. However, it will be of crucial im-

portance to add some modification to the current status of functional food science which is going for pharmacology. Food is quite different from medicine in various respects. It is necessary for implementation of functional food research to take into consideration the differences in terms of sensory properties. A new trend has come into being, which emphasizes the significance of studying the "secondary" function (see Figure 1.1). Since this *per se* is the most representative food attribute, even the science of functional food should consider taste and smell in a large measure. In the meantime, it was found that our body expresses taste receptors in the gut as well as in oral taste buds (Margoskee *et al.* 2007). Interestingly enough, it is possible that the gut's sweet taste receptor, T1R2-T1R3, functions to induce insulin secretion shortly after taking even artificial sweeteners and sweet protein (Shimizu-Ibuka *et al.* 2006). We now need some integrative studies on all the three categories of food functions (see Figure 1.1) and future research along this line will no doubt add a new dimension in the science and policy of functional foods.

1.4. DEFINITIONS AND LIMITATIONS

Currently, there is no general, worldwide definition of functional foods; however, there are multiple definitions developed by various organizations. The International Food Information Council (IFIC) states that functional foods "provide health benefits beyond basic nutrition (Hassler *et al.* 2004)." The limitation of this definition is that it lacks an explanation of how the food or what part of the food provides health benefits. The definition should differentiate whether the whole food or only food components are beneficial. The International Life Sciences Institute of North America (ILSI) states that functional foods are "foods that, by virtue of physiologically active food components, provide health benefits beyond basic nutrition (Hassler *et al.* 2004)." This definition is vague, but encompasses the realm of functional foods. There is no delineation between different types of functional foods, such as whole foods and foods that are enhanced with added ingredients. However, this definition provides insight into how the food or what part of the food is beneficial by stating "by virtue of physiologically active food components," (Hassler *et al.* 2004), this statement encompasses components of whole foods and components that are added to foods, which have beneficial effects. However, the statement about individual food components fails to demonstrate the role of synergy in providing

health benefits. The concept of synergy is important to include in functional food definitions because the coordination of the "physiologically active food component" with the other components present in the food is what provides the health benefit.

Health Canada states that functional foods are "similar in appearance to a conventional food, consumed as part of the usual diet, with demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions," (Hassler *et al.* 2004). One limitation to this definition is the narrowness of stating that a functional food must look similar to a conventional food. If a functional food appears similar to a recognizable food, consumer acceptance is likely increased; however, foods that do not appear similar to a conventional food should not be excluded since they may still act as functional foods. In addition, this definition is regional because appearance of conventional food differs according to location, culture, and religion. According to this definition, two foods may have the exact same physiological function and provide the exact same health benefit; however, if one of these foods has an unconventional appearance, it cannot be classified as a functional food. Another limitation to this definition is that the food must be "consumed as part of the usual diet," (Hassler *et al.* 2004). The usual diet of consumers varies from person to person, and a food should not lose classification as a functional food because it is not part of the usual diet of a consumer. If a functional food is part of the usual American diet, but not part of a usual European diet, is the same food not considered functional in Europe?

The Institute of Medicine of the National Academy of Sciences (IOM) states that functional foods are "those in which the concentrations of one or more ingredients have been manipulated or modified to enhance their contribution to a healthful diet," (Hassler *et al.* 2004). This definition does not differentiate functional foods from any other food; according to this definition, a reduced fat food can be a functional food (Katan and De Roos 2002). In addition, this definition only states that functional foods contribute to a healthy diet, not that they have specific functions regarding disease (Katan and De Roos 2002). Moreover, this definition excludes whole foods and foods that have foreign ingredients added; rather than only stating, "One or more ingredients have been manipulated or modified," the definition should include whole foods and functional ingredients that have been added to a food (Hassler *et al.* 2004).

In Japan, the FOSHU organization states that functional foods are

"processed foods containing ingredients that aid specific body functions in addition to being nutritious." The limitation to this definition is that all unprocessed, whole foods are excluded.

The American Dietetic Association (ADA) states that functional foods include "whole foods and fortified, enriched, or enhanced foods, have a potentially beneficial effect on health when consumed as part of a varied diet and on a regular basis, at effective levels," (Hassler *et al.* 2004). The limitation to this definition is that for a food to be functional, it must be "consumed as part of a varied diet," (Hassler *et al.* 2004). The statement about a varied diet was likely incorporated to impart the importance of an overall healthy diet; however, a functional food retains its functionality as part of any diet. The definition should be for a functional food, not a healthy diet.

The American Council on Science and Health states that functional foods are "whole, fortified, enriched, or enhanced foods that provide health benefits beyond the provision of essential nutrients, when they are consumed at efficacious levels as part of a varied diet on a regular basis," (Hassler 2002). The limitation of this definition is the same as the limitation of the definition offered by ADA.

1.5. RELEVANCE OF FUNCTIONAL FOODS

The concept of functional foods stems from the traditional paradigm of providing methods to prevent nutritional deficiencies; this paradigm encompasses foods that provide health benefits through fortification with micronutrients. Today, functional foods are designed to promote health by being targeted at specific physiological processes that may lead to disease prevention; this is known as the new paradigm. With the increase in diseases related to excess energy consumption, an aging population, and the increasing cost of health care and pharmaceuticals, consumers are turning to food to replace or augment traditional health care for health promotion and disease prevention. With increasing consumer interest in self-care of health, functional foods research will continue to expand as will the availability of functional foods to the consumer. The concept of functional foods needs to be incorporated into an overall healthy lifestyle for maximum benefit of disease prevention. Functional foods, an overall healthy diet including fruits, vegetables, unrefined grains, fish, and low-fat dairy products, and foods low in saturated fats and sodium (Katan and De Roos 2002), and exercise, combine to encompass the healthy lifestyle needed for disease

prevention. The definition of functional foods expands on one aspect of a healthy lifestyle; this definition is meant to recognize foods and food ingredients, whether natural, added, or modified, that provide disease prevention and health promotion benefits.

1.6. FUNCTIONAL FOOD VERSUS PHARMACEUTICALS

As the population ages, the incidence of chronic diseases associated with caloric excess and the cost of healthcare increases, and consumers increasingly desire self-care regarding health and an alternative to pharmaceutical management of disease (Hassler *et al.* 2004). Lifespan, incidence of obesity, and age are increasing, which results in an insurgence of chronic diseases such as cardiovascular disease (CVD) and diabetes mellitus. Each of these factors combines and occurs, leading to an increased cost of health care and pharmaceuticals. This increased cost of health care for disease management, coupled with pharmaceutical side effects, and consumer interest in self-care health management and healthy foods result in a demand for functional foods. Compounded to the expense of pharmaceuticals is their limited availability, since they are only available via prescription and their ineffectiveness for some consumers; moreover, consumers desire the preventable approach of functional foods over the symptomatic treatment approach of pharmaceuticals. The utilization of functional foods for health care encompasses the use of functional foods to replace or decrease dependence on pharmaceuticals. The use of functional foods rather than pharmaceuticals for health benefits will decrease healthcare costs, promote health without inducing pharmaceutical side effects, aid in disease prevention, and provide a method of self-care for consumers. In addition, the use of functional foods for health care has a broad spectrum due to food being necessary for survival, and the flexibility of foods for adaptation to suit specific demographic characteristics.

1.7. IMPACT ON HEALTH CARE AND SOCIETY

Functional foods are currently influencing healthcare through their incorporation into medical treatments, usually alongside pharmaceuticals. As the functional food industry expands, their use in healthcare will increase, allowing functional foods to act as a first line of defense against disease rather than pharmaceuticals. In addition, as functional food use for disease prevention continues, the incidence of chronic dis-

ease will decrease leading to a reduction in spending on health care and pharmaceuticals. Overall, the scope of healthcare will shift from treatment to prevention. However, an increase in the use of probiotics may exacerbate antibacterial resistance. Probiotic bacteria such as *Lactobacillus casei*, upon ingestion select for the survival and growth of *L. casei*, therefore "crowding out" other normal gut microflora. This gives probiotics antibiotic activity; some of the bacteria that are targets of this probiotic will survive, which leads to "super bugs" that are resistant to antibiotics. The use of probiotics and the excess use of pharmaceutical antibiotics compound to exacerbate the problem of antibiotic resistance. Overall, the shift away from pharmaceuticals and toward dietary intervention is necessary for health care in America; this shift will help to remove some of the governmental and societal burden of increasing health care costs, the inability to depend on social security benefits after retirement, and budgeted funds for Medicare.

1.8. FUNCTIONAL FOOD: SOURCES AND CLASSIFICATION

Functional foods are classified by source of origin, including plant, animal, microbial, and miscellaneous (algae, mushrooms, other). Regardless of the source of origin, the target of functional foods includes CVD, cancer, immune enhancement, gastrointestinal and women's health, aging, diabetes mellitus, and stress management.

1.8.1. Plant-Derived Functional Foods

Plant-derived functional foods are separated into primary and secondary metabolites; primary metabolites are plant compounds necessary for growth, while secondary metabolites are not essential for growth, but are used for plant survival mechanisms. Primary metabolites include plant proteins, beta-glucans, and omega-3 fatty acids. Plant proteins include texturized vegetable protein, soy protein isolate, and amino acids; these proteins act as functional foods by helping to decrease the amount of meat consumption, which decreases the consumption of fat and cholesterol. Beta-glucans, found in oats, act as functional foods by decreasing cholesterol absorption. Omega-3 fatty acids, found in flaxseed, act as a functional food by reducing platelet aggregation. Secondary metabolites include phytoestrogens, antioxidants, vitamins, tocopherols, steroids, gamma-linolenic acid (GLA), and phase II en-

zyme inducers. Phytoestrogens, estrogen-like compounds in plants, are found in soybeans and flaxseed and act as functional foods by decreasing post-menopausal cancer development. Antioxidants, such as anthocyanins, act as functional foods by quenching reactive oxygen species. Vitamins, which are abundant in fruits and vegetables, act as functional foods by preventing deficiencies; certain vitamins, such as vitamins C and E, also act as quenchers of reactive oxygen species. Tocopherols, which are vitamin E compounds found in oilseeds, act as quenchers of reactive oxygen species. Steroids are also found in oilseeds and act as functional foods by competing for cholesterol absorption. GLA is a fatty acid involved in the formation of prostaglandins and acts as an inflammatory modulator (4). Phase II enzyme inducers, found in Brassica vegetables, act as functional foods by glycosylating insoluble toxins to produce soluble compounds that are excreted. Consumption of foods containing phase II enzyme inducers also limits the phase I enzyme detoxification system; the phase I enzyme system produces reactive oxygen species.

1.8.2. Animal-Derived Functional Foods

Zoochemicals, which are animal-derived functional foods, include omega-3 and six fatty acids, conjugated linolenic acid (CLA), small peptides, whey and casein, and glucosamine and chondroitin sulfate. Omega-3 fatty acids include alpha-linolenic, docosahexaenoic (DHA), and eicosapentaenoic (EPA) fatty acids (4). Sources of alpha-linolenic acid include soy and canola oils, walnuts, and flaxseed (4). The main source of EPA and DHA is fatty fish, such as salmon. Omega-6 fatty acids include linolenic, gamma-linolenic, and arachidonic fatty acids (4). Sources of these fatty acids include some vegetable oils, nuts, and whole grains (4). Omega-3 and six fatty acids act as functional foods by enhancing immunity, modulating inflammation, and protecting against neurodegenerative diseases. CLA is a fatty acid present in milk that reportedly acts as a functional food by reducing cancer risks and adipose differentiation; however, a fatty liver may develop as a side effect (Hassler 2002). Whey and casein are milk proteins that act as functional foods by being easily digested and absorbed, and help build muscle mass; small peptides function in the same manner. Glucosamine and chondroitin sulfate are required for collagen formation and were stated to act as functional foods by alleviating pain associated with osteoarthritis; however, this claim has been disproved (4).

1.8.3. Microbial Functional Foods

Microbial-derived functional foods include probiotics, prebiotics, symbiotics, and synbiotics. Probiotics are natural microflora that occur in the gut, such as *L. casei* or numerous *Bifidobacter* species, which promote health (Hassler 2002). Prebiotics are dietary components that promote growth of probiotic bacteria. Symbiotics contain probiotics and prebiotics combined randomly, while synbiotics contain specific probiotics and prebiotics mixed together to benefit one another. Functional foods of microbial origin act by promoting the growth of probiotic bacteria so that the growth of pathogenic bacteria is limited.

1.8.4. Miscellaneous Functional Foods

Some functional foods are derived from miscellaneous compounds such as algae and mushrooms. Algae function by proving omega-3 fatty acids, which enhance immunity, modulate inflammation, and protect against neurodegenerative diseases. Functional foods derived from mushrooms contain antiviral, antibacterial, and anti-inflammatory properties.

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Prebiotics and Probiotics: Concepts and Advances

A.L. CARVALHO-WELLS and D.M.A. SAULNIER

2.1. INTRODUCTION

Any epithelial surface in the human body which is exposed to the external environment is subject to interaction with exogenous microorganisms. This occurs particularly on the respiratory, genital, and gut mucosal surfaces. The intestinal mucosa is the main site of interaction with the external environment and therefore is an important intermediary in the maintenance of health. The gut was traditionally thought of as a relatively simple organ comprising an epithelial tube surrounded by a layer of muscle. However recent research has unraveled the diverse functions of the gut and demonstrated the human gastrointestinal tract as a highly dynamic ecosystem, which is central to immune development, host defense, and human nutrition.

2.1.1. Location and Diversity of the Gut Microbiota

There are four main microhabitats in the gastrointestinal tract: the epithelial surface, the mucus layer which overlays the epithelium, the

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crypts (of the ileum, caecum, and colon), and the intestinal lumen, respectively. The levels and diversity of the microbial community within the gastrointestinal tract can differ according to the location as different physicochemical properties and substrate availability dictate the environmental conditions. Not surprisingly, the stomach mucosa is occupied by relatively few organisms due to the high level of acidity which requires a more acid tolerant organism; stomach contents (per gram) have been estimated to contain 10^3 colony forming units (CFU), reaching 10^4–10^7 in the small intestine and 10^{10}–10^{12} in the large intestine where the microbial numbers are highest (Holzapfel *et al.* 1998). The total area of the mucosal surface of the human gastrointestinal tract is 300 m^2 which makes it the largest surface area in the body that interacts with the external environment (Bjorksten 2006). The distal large intestine is the area of highest colonization with more than 500 different species (with some estimates suggesting up to 1,000 species) with potentially up to 100 billion microbial inhabitants (Boyle and Tang 2006). This enormous microbial community has been estimated to be in the region of 10^{14} microorganisms, the collective gene set of which is termed the "microbiome" and is thought to contain approximately 100 times the number of genes in the human genome (Backhed *et al.* 2005; Xu and Gordon 2003). Based on a meta-analysis covering several large Sanger-sequencing studies of human gut samples from different populations the most numerically dominant phyla within the intestinal microbial community are the firmicutes and bacteroidetes that comprise more than 90% of the total bacteria. Other bacterial phyla that have been isolated include also actinobacteria, gammaproteobacteria, verrucomicrobia, and betaproteobacteria (Hamady and Knight 2009).

Although the major dominant bacterial groups have been described, there is considerable species variation between individuals. For example, all humans possess several hundred species within each genus in their gut, however the foremost species within that genus will differ considerably between individuals (Simon and Gorbach 1984). The relationship between the human host and microbial symbionts is complex and a focus of modern biology. This has led to collaborative research projects the largest of which is probably the Human Microbiome Project, which is a worldwide strategy aiming to further delineate the extent of both human and microbial diversity. As it is a collaborative project the techniques used are sophisticated and will result in a vast data set, it is hoped that the project will be able to identify common features of the microbiome at a global level. This will help determine the impact

of host genetics on the development and stability of the microbiome by using genetically linked individuals evolutionary lineages (Ley *et al.* 2008; Turnbaugh *et al.* 2007).

2.1.2. Techniques Used to Elucidate the Commensal Microbiota

Microbiological techniques traditionally relied on the ability of a bacterium to grow on sterile semi or defined media in order to observe colonies. This enabled preliminary classification of bacteria on the basis of culture phenotype and biochemical characteristics. This was also the case for pioneering work in the area of gut microbiology; however, the majority of bacteria in the gut are anaerobes, therefore they present more of a challenge to grow in standard laboratory conditions. More recently, use of a culture-independent approach has revealed the complexity of the resident microbial communities; this was enabled by use of oligonucleotide probes based on 16S rRNA gene sequences. Further classification of bacteria based upon phylogenetic comparisons of the 16S rRNA sequences has been possible using qualitative and quantitative techniques. For example, PCR reactions and fluorescent in situ hybridization (FISH) use primers and probes respectively which target bacteria based on their 16S rRNA sequences. It is estimated that these specific probes have enabled identification of up to 80% of the total microbial diversity within the intestinal microflora, which would not have been possible with cultural techniques alone. Recent metagenomic techniques with high-throughput pyrosequencing have further explored functional genes encoded by the microbiome using annotation schemes such as Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways or Clusters of Orthologous Groups (COGs) (Turnbaugh et al., 2006, 2007, 2009). Molecular analysis has shown that the aerobic species present reach relatively high cell densities and metabolic activity in the human caecum, in fact 50% of total bacterial ribosomal RNA was found to correspond to these species in this region of the gut. This is in contrast to feces in which only 7% of the total bacterial rRNA from these species is found (Marteau *et al.* 2001).

2.1.3. Factors Affecting the Composition of the Gut Microbiota

In a healthy gut, there is a balance between potentially harmful, commensal and beneficial bacteria. Probably the most important factors in determining the initial colonization pattern are the type of delivery at

birth (either vaginal or caesarean section) and the initial diet (whether the newborn is fed mother's milk or infant formula). The newborn microbiota changes rapidly during the first few weeks and during weaning (Boyle and Tang 2006). Other important factors include the environment, age, gender, and diet (Santosa *et al.* 2006). Differences in microbiota composition have been found between infants born in different countries and raised with different diets and even between hospital wards (Adlerberth *et al.* 1991; Lundequist *et al.* 1985; Santosa *et al.* 2006; Simhon *et al.* 1982). The composition of the adult microbiota is thought to be more stable, however acute effects can disrupt this homeostasis for example, during antibiotic treatment, after gastrointestinal surgery, exposure to radiation, and in some infectious (diarrheal) disease states (Mombelli and Gismondo 2000). The stability of the microbiota is compromised in the elderly, demonstrating significant variability over time and between individuals; as well as lower species diversity (Claesson et al., 2011; Makivuokko et al, 2010).

2.2. ROLES OF THE COMMENSAL MICROBIOTA

The presence of the gut microbiota has influenced human evolution in that the human host cannot perform certain vital intestinal functions without them. Germ-free animal models have provided useful insights into the roles of the microbiota and the extent of interaction between the host and the gut microbiota. It can be thought of as a 'microbial organ' as the processes performed by this diverse population are extensive; it can communicate with itself (bacteria: bacteria) and with the host (bacteria: human). Maintenance of homeostasis is an interactive process between the bacteria and host epithelia which influences both intestinal physiology and the derivation and distribution of energy. Landmark studies in recent years have shown that the composition of the microbiome can have an impact on energy balance and obesity (Turnbaugh and Gordon 2009; Turnbaugh *et al.* 2006).

2.2.1. Fermentation

A major role of the microbiota is to ferment nondigestible dietary components and endogenous mucus produced by the gut epithelium. This is an example of a symbiotic relationship as the human host benefits from a wide array of microbial enzymes which are outside the host's own biochemical repertoire. This provides the host with a source of

energy from the food ingested and also to the microflora which in turn is used to sustain the microbial community. Fermentation differs according to the site within the gut as shown in Figure 2.1. The major source of energy from colonic fermentation is from carbohydrates, which include large polysaccharides (such as plant derived pectin, hemicellulose, cellulose, gums, and resistant starch) and also less complex carbohydrates such as oligosaccharides and nonabsorbed alcohols and sugars (Cummings *et al.* 1996; Cummings *et al.* 1987). Fermentation is not limited to carbohydrates but also other dietary components such as proteins and glycoproteins.

2.2.2. Short Chain Fatty Acids

The main fermentation end products are short chain fatty acids (SCFA) which are small organic molecules absorbed by diffusion; carriers mediated exchange or ion exchange processes. Common examples include acetate, butyrate, and propionate, and are considered mainly as compounds which provide a source of energy to the host. Fermentation activity differs according to area within the gut, the most metaboli-

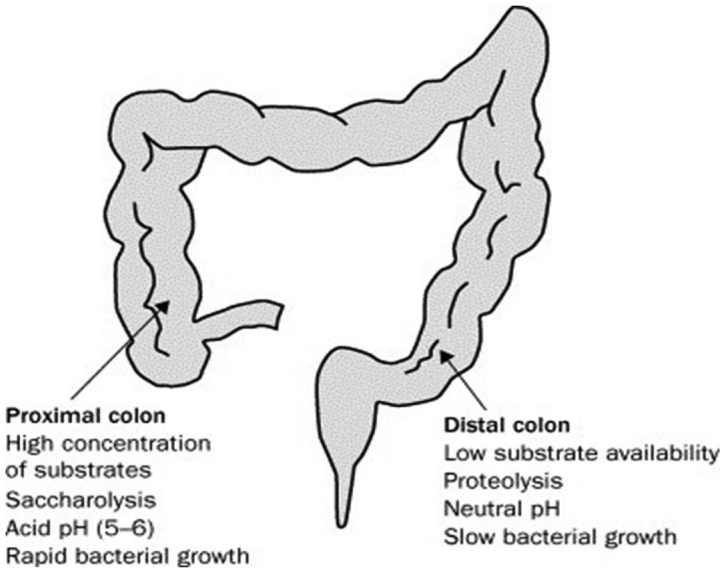

FIGURE 2.1. *Fermentation in the colon. Fermentation activity differs according to the specific location within the gut. The most metabolically active area is the caecum and right (proximal) colon. The left side has a different pH which favors a more proteolytic fermentation (Gibson and Roberfroid 1995).*

cally active area is the caecum and right colon, consequently this is an area of rapid bacterial growth, low pH (5–6) and rapid generation of SCFA as a result of carbohydrate fermentation (Cummings *et al.* 1987; Macfarlane *et al.* 1992). Fermentation can also occur with noncarbohydrate substrates, therefore proteolytic fermentation (of proteins and peptides) also generates potentially damaging compounds such as ammonia, amines, and phenolic compounds (Macfarlane *et al.* 1986). In contrast, the left side of the colon has less carbohydrate fermentation, the pH is less acidic, and it is associated with an increase in proteolysis which has been linked to the production of harmful nitrogenous products, which accounts for the recommendation to eat a carbohydrate and fiber enriched diet (Guarner and Malagelada 2003). The absorption of ions such as calcium, magnesium, and iron in the caecum is improved in the presence of SCFA (Roberfroid *et al.* 1995; Younes *et al.* 2001). The presence of adequate SFCA causes colonic water and sodium ions to be absorbed, therefore producing solid stools therefore having an effect on intestinal transit time, although the mechanisms are unclear (Elsen and Bistrian 1991). SCFA are not only a source of energy for tissues but can also have important effects on host physiology as one SCFA in particular, butyrate has been shown to inhibit the development of colonic cancer cells in vitro (Pool-Zobel and Sauer 2007a). The colonic epithelium almost entirely consumes the butyrate that is produced as it is a preferred energy source (Cummings *et al.* 1987). SCFA have a positive effect on epithelial cell differentiation and proliferation in vivo. Some members of the gut microbiota also produce vitamins such as folate, biotin, and vitamin K-2 (Conly *et al.* 1994; Hill 1997).

2.3. THE GUT MICROBIOTA AND AUGMENTATION OF HOST DEFENSE

2.3.1. Barrier Function

One role of the commensal flora is to protect against infection from exogenous organisms, of which there is a higher risk within the gastrointestinal tract. Several mechanisms are thought to contribute to this process, which is collectively termed colonization resistance. The important role of the commensal microbiota in boosting host defense has been confirmed in germ-free animals which demonstrate an increased rate of susceptibility to infections relative to a wild type microflora (Baba *et al.* 1991; Taguchi *et al.* 2002). Adhesion is thought to be a

CHAPTER 7

Extraction and Purification of Bioactive Ingredients from Natural Products

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7.1. INTRODUCTION

Bioactive compounds are expected to play an important role as one of the major sources of new drugs in the years to come because of their incomparable structural diversity, the relatively small dimensions (< 2000 Da), and their "drug-like" properties, i.e., their ability to be absorbed and metabolized [1]. Isolation of natural products from higher plants, marine organisms, and microorganisms is critical, using state-of-the-art methodologies. The plant kingdom contains approximately 80–100,000 plant bioactive compounds and the separation and isolation processes are cumbersome and tedious. Isolation of natural products generally combines various separation techniques, which depends on the solubility, volatility, and stability of the compounds to be separated. The choice of different optimization parameters of separation is critical and essential

7.2. EXTRACTION OF BIOACTIVE INGREDIENTS

Isolation of bioactive ingredients from the plant materials is a trial and error excise in which different solvents are tried under a variety of conditions such as time and temperature. The main objectives of the

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extraction and purification of unique/unstudied bioactive components, exploring the potential of secondary metabolites for chemical fingerprinting or metabolomics studies, and bioassay derived identification of bioactives.

Isolation of bioactive compounds involves dissolution of solutes from the plant matrix, diffusion of the compounds into the extractant, and separation of solutes using chromatographic techniques.

7.2.1. Selection of Solvent

In choosing a solvent for the extraction of bioactives, the ability to extract components has to be considered. For instance, ionic solutes can be extracted from aqueous solvents. The general features of the bioactive molecule that are helpful to ascertain the isolation process include partition coefficient, acid-base properties, charge, stability, and molecular size. In literature, many basic extraction procedures are available [2]. Solvent choice for the extraction is a critical step. Single solvent is unlikely to extract all groups of bioactive compounds from the natural plant materials. In most of the cases, these methods will be refined to our requirements in terms of plant materials and solutes of our interest. The expected outcome from this extraction process should be high purity product, adequate quantity of bioactive compound, and confirming the stereochemistry of the molecule.

In general, three conventional methods were used for the extraction of bioactive compounds such as solvents, steam, and supercritical fluids. On a global level, water extraction is practised while making coffee or tea. Basically, pretreated plant material is extracted with hot water which takes up the flavor, taste, and color of the components. After filtration, the extract is ready for consumption. In case of the isolation of certain bioactive compounds from plant material by means of liquid extraction, some technological problems needs to be resolved [3]. First the plant material has to be pretreated in order to obtain reasonable extraction yields. Another problem is the need for special solvents to be used in the extraction procedure [4]. More recently, attention has been focussed towards the isolation of specific compounds that can be used in the food industry. Of particular interest is the isolation of bioactive compounds, aromas, and fragrances from plants and fruits [5,6]. The sequential extractions of bioactives using nonpolar to polar solvents are depicted in Figure 7.1. Various polarity solvents are reported as follows: (1) nonpolar solvents (hexane, heptanes, petroleum ether,

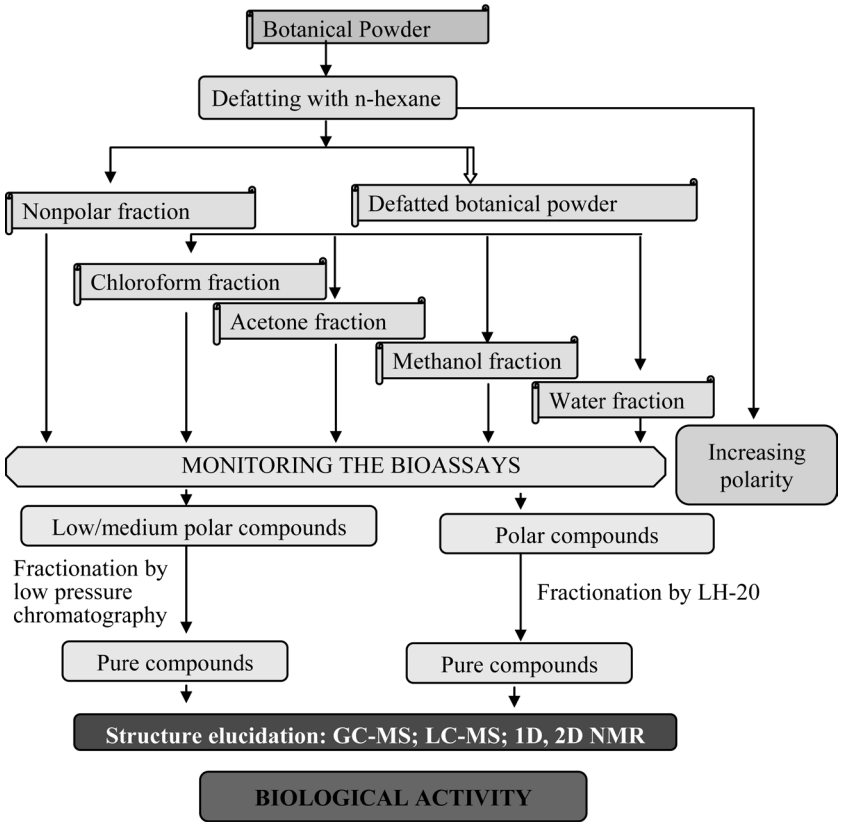

FIGURE 7.1. *Schematic flow sheet of bioassay directed isolation, characterization of botanicals by successive extraction with different solvents.*

benzene, and toluene); (2) medium polar solvents (dichloromethane, chloroform, ethyl acetate, acetone, and methyl ethyl ketone); and (3) polar solvents (methanol, ethanol, acetonitrile, and water).

7.2.2. Selection of Raw Materials

In a typical extraction process, the selection of raw material is critical. For instance, extraction of compounds sensitive to heat and light such as carotenoids needs fresh raw materials, while certain fatty acids, sterols, and phenolics can be isolated from dried material. Thus, drying and grinding of plant material or homogenization of fresh raw material is critical. Prior to choosing a method, it is necessary to establish the main objective of the extraction [7,8].

7.2.3. Fractionation

After extraction from the plant, the bioactive components have to be separated from the crude mixture. It involves further solvent partitioning and extensive chromatography by retaining their properties of the desired compound, such as acidity, alkalinity, polarity, stability, molecular size, and structure. This may involve simple crystallisation of the compound from the crude extract, e.g., isolation of the glycoside dianellin from *Dianella caerulea* [9] as well as crystallization of limonin from citrus extract [10]. In some cases, the isolation can be assisted by the preparation of suitable derivatives, imparting more easily manageable properties of the desired compound, e.g., isolation of the swainsonine as its triacetate from *Rhizoctonia leguminicola* and isolimonoic acid was isolated as methyl isolimonate [11]. Finally, purification will be performed to provide compounds of high purity for structural analysis, which may be accompanied by appropriate techniques such as crystallisation, sublimation, or distillation [12]. According to Pettit et al., the isolation of bioactive compounds from natural sources is, "always challenging and every step require judgement, improvisation to discover novel components," [13].

7.3. CONVENTIONAL EXTRACTION TECHNIQUES

Traditionally-used techniques for the extraction of bioactive compounds are discontinuous, continuous, and hybrid approaches. The discontinuous techniques include the use of either organic solvents (sometimes assisted by ultrasound) or water, while steam distillation and vacuum distillation are continuous methods. Some methods involving both continuous and discontinuous approaches, such as distillation–extraction; Soxhlet extraction has also been reported [14].

Solvent extraction is a traditional method for extracting bioactive compounds from different plant materials [15–17]. However, less polar components present in most plant tissues may interfere with the subsequent separation [17,18]. The conventional methods for the extraction of natural products are Soxhlet extraction, cold percolation, hot extraction, and sonication. These methods have certain drawbacks, including long extraction time, use of large amounts of organic solvents, unsatisfactory extraction efficiency, and potential degradation of labile compounds. Therefore, the sequential solvent extraction is recommended for efficient separation of bioactive compounds [19]. For this reason,

lipophilic compounds are removed with nonpolar organic solvents such as hexane or dichloromethane [17, 20–23]. Meanwhile, the hydrophilic constituents are extracted with polar solvents such as acetone, methanol, ethanol, and water [21,24,25].

In some cases, the addition of a polar solvent, such as water, to the sample may increase the recovery of more polar compounds [24,26]. Meanwhile, some bioactive compounds of low or medium polarity can be extracted efficiently with more nonpolar solvents [27–29]. Direct extraction of bioactive compounds of a low polarity can also be obtained with polar solvent at high temperature [18,30]. However, the subsequent cleanup step to separate the low polar bioactive compounds from the polar extract is a challenge [31].

7.3.1. Continuous Techniques

This technique involves steam distillation along with solvent extraction for the isolation of essential oils from plants. This technique has been applied extensively as a step prior to compositional studies of essential oils, such as curcuma [32], marjoram [33], grapes [34], soybeans [35], and lavender [36].

7.3.2. Discontinuous Techniques

Solvent extraction has long been used for the isolation of essential oils from natural products. This technique uses either pure organic solvents or mixtures. Organic solvent extraction assisted by ultrasound, also known as sonication, is another technique widely used for the isolation of essential oils/extracts from plants. Thus, sonication methods based on 20–30 minutes of extraction with methanol–chloroform mixtures have been used for the isolation of white clove essential oil [37] and cyanogenic glucosides [38].

The use of organic solvent extraction has certain limitations such as solvent residues in the extract, with the subsequent toxicological risk and the long extraction time required in most cases for achieving efficient extractions. In addition, organic solvents have a low selectivity. Thus, apart from the desired substances, high molecular weight, nonvolatile components, such as fatty oils, resins, waxes, and coloring matters, are coextracted. The nonfeasibility of automation of the technique is another important drawback to be taken into account.

Water extraction (under ambient conditions, without the application

CHAPTER 11

Mechanism of Neuroprotection by Bioactive Compounds

R.C. STAVINOHA, B.Y. JAMISON, Y. GOMADA, V. MAITIN and D.A. VATTEM

11.1. INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) are the two most prevalent neurodegenerative diseases worldwide [1]. These devastating diseases are characterized by progressive and irreversible neurodegeneration of particular neuronal networks in the brain that lead to severe cognitive and behavioral dysfunction [1]. The etiology behind the development of these diseases remains elusive [2]. Although genetic susceptibility has been identified in both PD and AD, the majority of cases are sporadic and without certain cause. It is thought that aging, oxidative stress, and environmental factors are involved in the initiation of the diseases; however, the molecular mechanisms that underlie the pathogenesis are not clear. The current treatments for AD and PD offer symptomatic relief, but do not affect the underlying neurodegeneration or natural course of the disease [2]. As such, the development of treatments that can slow or reverse the pathological processes of these diseases is currently a significant focus of research.

11.2. ALZHEIMER'S DISEASE

AD is the most common neurodegenerative disease in the world. At

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present, the disease affects 24% of the population over age 85.3 In the year 2000, it was estimated that 4.5 million individuals in the United States had AD, a number that is projected to rise to 14 million by 2050 [3]. This disease involves pathological events that affect the anatomy, biology, and function of selective regions of the brain [4]. Specifically, widespread neurodegeneration is observed in the cortex and hippocampus [5]. AD is clinically characterized by a progressive loss in cognitive function that typically begins with memory loss, anxiety, and depression. As the disease progresses, the symptoms evolve to severe motor dysfunction, profound cognitive deterioration, and loss of independent function [4]. Pathologically, AD involves a cascade of events that lead to modifications in the metabolism of the amyloid precursor protein (APP) and the tau protein [4]. These changes result in the characteristic development of extracellular amyloid beta-protein (Aβ) deposits and intraneuronal neurofibrillary tangles (NFTs), respectively [4,3]. Consequently, common cell signaling pathways are affected and result in neuronal network dysfunction, neuronal loss, neurotransmitter failure, and cell death [4].

11.2.1. Amyloid Beta-Protein Deposits

The APP is a transmembrane protein expressed ubiquitously in cells, indicating that it has a normal biological role; yet, its function is unknown [6]. In cases of AD, the APP is found to be aberrantly processed to yield soluble Aβ oligomers and insoluble Aβ fibrils that aggregate extracellularly in the brain [6]. The metabolism of APP normally occurs through a cleavage by α-secretase, a membrane-bound protease that acts on APP within the Aβ domain, therefore it does not produce the Aβ peptide [4,7]. For unknown reasons, in AD, APP is processed in an alternate pathway that involves two subsequent cleavages [7]. In this pathway, referred to as the amyloidgenic pathway, the enzyme α-secretase first cleaves APP, releasing a membrane bound C-terminal fragment consisting of 99 amino acid residues, referred to as C99 [4]. Gamma-secretase subsequently cleaves C99 and releases Aβ, typically containing 40, 42, or 43 amino acids, into the transmembrane domain [4]. The Aβ peptide can oligomerize to form soluble oligomers, but may also converge to form insoluble fibrils in a beta-sheet conformation that are deposited extracellularly [7]. It has recently been found that the soluble Aβ oligomers accumulate in synapses and behave as a pathogenic ligand to membrane proteins, which disrupts synaptic transmission and

downstream events that are required for memory formation [7]. The insoluble Aβ fibrils that form are deposited as plaques extracellularly and contribute to neurodegeneration in several ways. Aβ plaques are thought to directly contribute to synaptic dysfunction by altering synaptic plasticity [4]. The plaques are thought to promote oxidative stress by decreasing antioxidant enzymes, increasing free radical production, and/or causing mitochondrial dysfunction [3]. Additionally, inflammation surrounding the plaque is believed to promote the degeneration of nearby neurons [4]. Aβ plaques are also thought to disrupt cellular functions by interacting with cell membranes and promoting oxidation [6]. Furthermore, Aβ plaques form poorly selective channels in the lipid bilayer that disrupts the membrane potential responsible for generating action potentials, which results in an influx of calcium into the cell that promotes apotosis [6].

11.2.2. Neurofibrillary Tangles (NFTs)

NFTs are intraneuronal protein aggregates primarily composed of the hyperphosphorylated cytoskeletal protein, tau. The main function of tau relates to microtubule stability. Tau is intimately involved in maintaining the balance between assembly and disassembly of the microtubules, a function that is essential to the stability of the cytoskeleton and to the integrity of neurons [6]. The phosphorylation state of tau modulates the stability of the microtubules and is regulated by various protein kinases and phosphatases. However, in the case of AD, tau is found to be aberrantly hyperphosphorylated, rendering it incapable of microtubule interaction, consequently impacting normal neuronal functions, morphology, and viability [6]. Recent evidence suggests that, in addition to being a microtubule stabilizer, tau may regulate neuronal excitability and serve as a master regulator of the trafficking of molecules within the cell that contribute to synaptic function [8]. These additional functions are also disrupted by the hyperphosphorylation of tau and contribute to the dysfunctional regulation of neuronal signaling and synaptic function common to AD. This is evidenced by a strong correlation between the hyperphosphorylated tau and a decrease in presynaptic protein expression [9]. Interestingly, studies have shown that reduction in the levels of tau prevents the neurotoxic effects of Aβ oligomers, suggesting that the damage induced by Aβ oligomers on the synapses may be mediated by the aberrant phosphorylation of tau. In addition, both oxidative stress and soluble Aβ oligomers are believed to promote the

actions of the kinases ERK, p38, and JN, which are capable of phosphorylating tau [8].The hyperphosphorylation of tau promotes its aggregation and represents the main component of the insoluble NFTs characteristic of AD [4].

11.2.3. Pathological Mechanisms

Convincing evidence reveals that oxidative stress plays a key pathogenic role in the dysfunction and death of neurons that drives neurodegenerative diseases. Free radicals are highly reactive and unstable molecules formed constantly in cells and must be neutralized by an antioxidant defense system to maintain redox homeostasis [1]. Oxidative stress occurs when there is an imbalance between the production of free radicals and the ability of the cells to neutralize these toxic compounds [1]. When redox homeostasis is lost, the overabundant free radicals react with and damage cellular components with destructive consequences, such as lipid peroxidation, enzyme inactivation, nucleic acid breakage, and altered membrane fluidity [6]. Oxidative stress can cause damage to all biomolecules and eventually leads to cell death, if not resolved [6]. The brain is particularly susceptible to conditions of oxidative stress since it consumes approximately 20–30% of the oxygen inspired [5]. Ninety percent of free radicals formed in the cell are produced by the mitochondria, an organelle that neurons are highly reliant on for oxidative phosphorylation and the maintenance of membrane polarity [1]. Additionally, the brain contains a high concentration of polyunsaturated fatty acids (PUFAs) that are particularly vulnerable to oxidation [5]. Although oxidative stress is known to be a key factor in AD pathology, it remains unclear if it is a cause or result of the disease [6]. Evidence suggests that oxidative stress promotes the formation of Aβ plaques and NFTs; however, it has also been observed that the presence of these protein aggregates promotes oxidative stress [6]. This relationship creates a detrimental cycle of free radical production that overwhelms the antioxidant defense system [6]. Many proteins have been identified that are damaged by oxidative stress in AD, including proteins involved in cell signaling, neuronal communication, antioxidant defense, regulation of neurotransmitters, pH regulation, energy metabolism, phosphorylation of tau, and processing of APP [5]. The oxidative damage to these important proteins disrupts their activity and contributes to the molecular dysfunction seen in AD [5]. An increase in lipid peroxidation has also been observed in the brains of individuals

with AD [6]. The peroxidation of the particularly susceptible PUFAs in the cell membrane result in alterations in the membrane composition and is thought to contribute to the decreased expression of the nicotinic acetylcholine receptors commonly seen in AD [6]. In addition, byproducts of lipid peroxidation such as HNE and acrolein are capable of covalently modifying proteins and inhibiting enzyme activity [6]. HNE has also been shown to upregulate the expression of β-secretase, thus promoting the processing of APP to form Aβ peptide [6]. Oxidative stress can also cause modifications to DNA, including strand breakage, cross-linking, base modification, and oxidation of deoxyribose [1]. Oxidative damage to mitochondrial DNA further promotes oxidative stress by causing modifications in the electron transport chain that lead to the leakage of electrons [6].

Originally, inflammation was thought to be a consequence of neurodegeneration, but recent evidence suggests that it may also be a primary pathogenic factor [2]. The persistent formation of plaques and progressive neuronal damage that occurs leads to the overactivation of microglia, the immune cells of the central nervous system. The overactive microglia release large amounts of proinflammatory and cytotoxic factors, which causes damage to the neurons and further activate microglia, creating a vicious cycle of uncontrolled and prolonged inflammation which drives neurodegeneration [10]. Excitotoxicity is another pathological factor common to many neurodegenerative diseases, including AD. Excitotoxicity results from the excessive activation of N-methyl-D-aspartate (NMDA) receptors by glutamate; as a result, calcium levels in the cell increase and triggers apoptosis [11].

11.2.4. Risk Factors

The greatest risk factor for the development of AD is age [3]. It is thought that the increase in oxidative stress that occurs with aging constitutes a large part of the risk. Vascular diseases, such as hypertension, hypercholesterolemia, and diabetes, also increase the risk of AD [3]. On top of vascular complications, diabetes poses an additional risk for AD due to the effects of hyperinsulinemia and insulin resistance on amyloid metabolism [3]. This correlation is evidenced by a significantly high incidence of AD development among individuals with diabetes. It has been suggested that hyperinsulinemia and insulin resistance leads to decreased clearance and increased deposition of the Aβ peptide [12]. Additionally, impaired insulin-signaling results in abnormal glucose

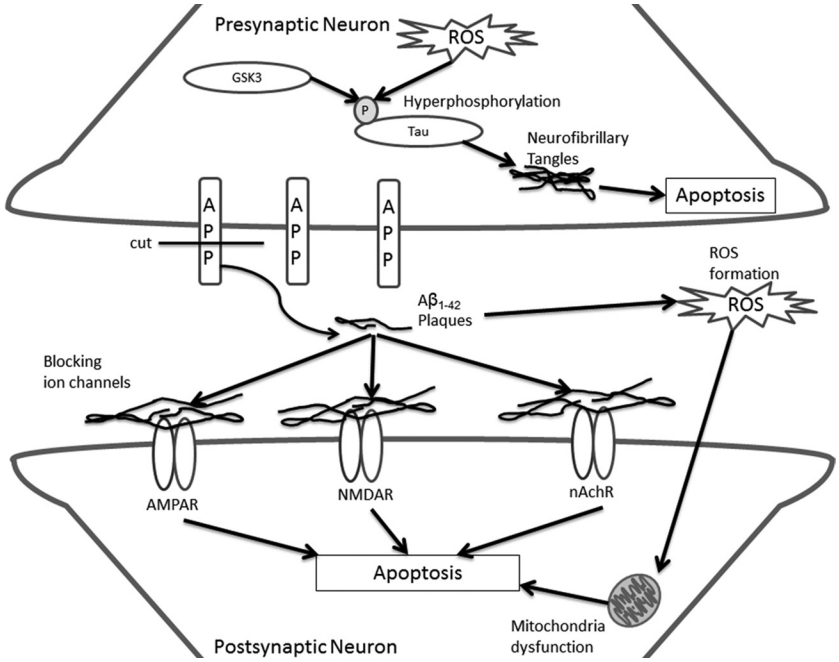

FIGURE 11.1. Possible molecular mechanisms behind development of Alzheimer's Disease.

and energy metabolism, oxidative stress, mitochondrial dysfunction, and hyperphosphorylation of tau via increased activity of glycogen synthase kinase 3 [7]. Although the majority of cases of AD are sporadic and without certain cause, approximately 10% of cases can be ascribed to genetic susceptibility [6]. Mutations in APP, presenilin 1, and presenilin 2 located on chromosome 21, 14, and 1, respectively, have been identified to promote the abnormal processing of APP that leads to aggregation [6]. Additionally, inheritance of the ε4 allele of the apolipoprotein E gene on chromosome 19 increases the risk for the development of AD [6].

11.3. PARKINSON'S DISEASE

PD is the second most common neurodegenerative disease that affects 0.3% of the population of industrialized countries [2]. The prevalence increases to 1% of the population over 60 years of age, and to 4% of the population over 80 years of age [2]. PD involves a progressive loss of neurons in the brain manifesting into characteristic motor and

non-motor symptoms. Motor symptoms include akinesia, bradykinesia, rigidity, resting tremor, postural instability, and gait impairment [11]. Nonmotor symptoms include anosmia, depression, anxiety, sleep disorders, gastrointestinal symptoms, autonomic dysfunction, and cognitive impairment [11].

11.3.1. Dopaminergic Pathway and Lewy Bodies

PD is a neurodegenerative disease of the central nervous system hallmarked by the degeneration of dopaminergic neurons, dopamine deficit, and the presence of Lewy bodies in remaining neurons [13]. Although there are signs of pathology in multiple neuronal systems in PD, the cardinal symptoms developed are ascribed to the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and subsequent loss of the neurotransmitter dopamine [10]. The SNpc is critical for the control of motor function, since it promotes voluntary movement and inhibits involuntary movement by affecting the basal ganglia loops, a process mediated by dopamine receptor D1 activation and D2 inactivation [10]. Neurodegeneration in this dopaminergic pathway leads to dopamine depletion, which has shown to alter the proportions of the D1- and D2-receptors [14]. Specifically, the loss of dopamine results in reduced activity of the D1-receptor and increased activity of the D2-receptor [14]. These pathological changes in the dopamine receptors alter the balance in the basal ganglia loops and result in the characteristic motor symptoms of the disease [10]. Lewy bodies are found in the cytoplasm of the remaining neurons of the SNpc; however, they are also found in other nondopaminergic neurons, including neurons of the amygdala, basal nucleus of Meynert, hippocampus, and brain stem [10]. The presence of Lewy bodies in these nondopaminergic neurons indicates that they play a role in the development of the nonmotor symptoms associated with PD [2]. Alpha-synuclein (α-syn) has been identified as the primary protein aggregate found in Lewy bodies [11]. The exact cause of aggregation is unclear; however, overexpression of the protein, genetic mutations, and oxidative stress are thought to contribute [11]. Alpha-syn has been identified as a key factor implicated in PD, because it plays an important role in controlling the function of dopaminergic neurons; thus, the dysfunction of this protein at dopaminergic synapses is thought to contribute to the initiation of neurodegeneration [15]. Additionally, it is suggested that the aggregation of α-syn has a pathogenic role in PD through exerting

negative effects on the cell membrane and proteasomal functionality, disrupting gene expression regulation, influencing cell signaling and cell death pathways, promoting inflammation, and modifying the storage and release of dopamine [11].

11.3.2. Pathological Mechanisms

No single pathogenic event has been found to be the primary factor in the development of PD [11]. Rather, the process appears to be multifactorial involving several mechanisms that act synergistically in a complex manner to foster neurodegeneration [11]. Similar to AD, mechanisms involved in the pathogenesis of PD include oxidative stress, inflammation, and excitotoxicity [11]. Oxidative damage to lipids, proteins, and nucleic acids has consistently been observed in the SNpc of individuals with PD [11]. The cause of increased oxidative stress in PD is unclear, but it has been suggested that mitochondrial dysfunction and accelerated dopamine metabolism play a role [10]. The exact cause of mitochondrial dysfunction in PD is also unknown, but results in further production of free radicals, presumably by defects in the electron transport chain that lead to the leakage of electrons [10]. Dopamine metabolism is accelerated in PD, and contributes to oxidative stress through the concurrent production of free radicals such as quinones and peroxides [10]. The vulnerability of dopaminergic neurons to neurodegeneration in PD compared to other neuronal systems may be linked to unique morphological and physiological properties [10]. The dopaminergic neurons consist of long, narrow, branched projections that have higher basal energy requirements than other neurons, rendering them more susceptible to damage caused by mitochondrial dysfunction [10]. Additionally, dopaminergic neurons are particularly susceptible to oxidative stress due to a high rate of oxygen and calcium metabolism, elevated iron concentrations, and reduced levels of antioxidants, specifically glutathione [10]. Inflammation is another mechanism involved in PD, as evidenced by microglia activation in the striatum and elevated levels of proinflammatory cytokines in the cerebrospinal fluid and basal ganglia in individuals with PD [11]. Microglia can become activated in PD in response to toxins, protein aggregates, or damaged neurons and become chronically active as a result of positive feedback from dying neurons, as seen in AD. Excitotoxicity is also implicated in PD. Dopaminergic neurons have particularly high levels of receptors for glutamate, the principle excitatory neurotransmitter of the

central nervous system, rendering them vulnerable to glutamate-mediated toxicity [11]. As with AD, over activation of the NMDA receptors by glutamate leads to increased intracellular calcium levels, which promotes apoptosis [11].

11.3.3. Risk Factors

The etiology behind PD is largely unknown, but there are some genetic and environmental factors identified that contribute to the development of the disease [10]. Similar to AD, age represents the biggest risk for developing PD [11]. Environmental factors, such as the neurotoxin MPTP and certain pesticides are known to cause PD through the inhibition of complex 1 in the mitochondria [1]. The active metabolite of MPTP, MPP^+, exerts toxic effects on the dopaminergic neurons causing mitochondrial membrane potential impairment, altered calcium handling, excessive free radical production, and ultimately cell death [11]. Rotenone is an example of a pesticide that also exhibits detrimental toxic effects on complex 1 in the mitochondria [2]. Other environ-

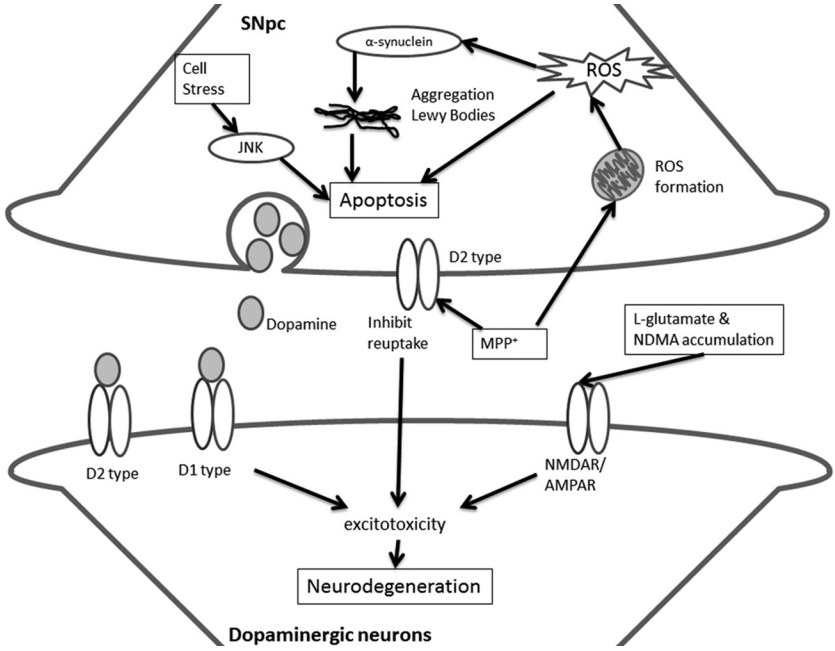

FIGURE 11.2. *Possible molecular mechanisms behind development of Parkinson's disease.*

mental risk factors include carbon monoxide poisoning, exposure to solvents, and hydrogen sulfide intoxification [2]. Although rare, various genetic mutations have been identified to cause PD in about 5–10% of cases [16]. Specifically, mutations in α-syn, parkin, DJ-1, and LRRK2 are associated with the development of PD [1]. These mutations are believed to cause mitochondrial dysfunction and increased oxidative stress, and thus contribute to the development of PD [1].

A recent study reported that dysfunction of p38 or JNK of MAPK signaling is involved in development of AD, PD, and amyotrophic lateral sclerosis (ALS) due to its effects on development and apoptosis [17]. It is reported that overexpression of p38 in microglia, astrocyte and neuron may increase inflammation, excitotoxicity, and synaptic plasticity via upregulation of TNF-α, IL-1β, and nitric oxide [18]. Also, accumulation of amyloid β plaques and overexpression of p38 were reported to involve tau phosphorylation, thus it is suggested that p38 MAPK signaling dysfunction may contribute to PD [18]. Also, activation of p38 MAPK signaling and inflammation via NF-κB was observed in a MPTP-treated mice brain, and it is reported upregulation of JNK mediated by ASK1 and MAPK increase neuronal cell death, thus, p38 MAPK is considered to play important role in PD. Also, p38 can phosphorylate GSK-3 along with PI_3K/AKT, p38 MAPK is shown to be involved in axonal regeneration [19]. As described, the activity of p38 MAPK regulate many cellular functions, however, during several pathologies, p38 MAPK is shown to be hyperactive, which has results in the progression of neurodegenerative disease, therefore proper regulation of MAPK signaling may become a therapeutic target of neurodegenerative disease. In an AD patient brain, reduced TGF-β signaling are seen and suggested to progress formation of neurofibrillary tangles of aggregates of Aβ, especially $A\beta_{1\text{-}42}$ [20]. It is reported that the reduction of TGF-β signaling reduces trophic support of neuron from glial cell and also reduces anti-inflammatory proteins, thus it is considered to promote the progression of AD [21]. However, overexpression of TGF-β signaling is reported to increase neuronal apoptosis and reduce phagocytosis, thus increases accumulation of aggregated Aβ which may contribute to AD (Lee *et al.* 2010). Also, reduction of TGF-β is reported to trigger apoptosis and is suggested to contribute to the development of PD due to increase of ROS formation of microglia [23]. Activation of TGF-β in macroglial cells attributes to inhibit PHOX induced ROS production via prevention of Erk activity, thus activation of TGF-β may play an important role in preventing PD progression through reducing

CHAPTER 15

Therapeutic Potential of Green and Black Tea in the Prevention and Treatment of Various Diseases

A. SHEHZAD, M. UL-ISLAM, N. SHAH and Y. SUP LEE

15.1. INTRODUCTION

Tea, derived from *Camellia sinensis*, is one of the most widely consumed beverages in the world. It is cultivated mostly in China and India, as well as parts of some Asian and African countries [1,2]. Tea has received interest from both scientific and consumer communities for its health benefits. Tea is rich in substances with antioxidant properties and contains small quantities of proteins, carbohydrates, amino acids, vitamins, and minerals [3]. Currently, high levels of tea drinking throughout the world are attributed to its biological functionality, rather than to habitual tea drinking, which is generally driven by its flavor and stimulant effect. The presence of polyphenolic compounds has given tea impressive therapeutic properties, including antioxidant, anti-inflammatory, antitumor, and metabolic regulatory effects [4].

Although originating from the same plants, tea is categorized into three main types based on their levels of oxidation [5]. Green and black teas are processed differently during manufacturing. To produce green tea, the freshly harvested leaves are steamed and dried to inactivate the

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polyphenol oxidase enzyme. This process retains the polyphenols in their monomeric form. In manufacturing black tea, the tea leaves are allowed time for extended fermentation, allowing polyphenol oxidase to catalyze the catechin polymerization, resulting in the formation of polymeric compounds [4]. The composition of black tea is thus dependent on the production process. Oolong, a third type of tea, results from the partial fermentation of tea leaves, and represents a mixture of green and black tea compounds.

Both green and black teas are rich in flavonoids, which are water soluble pigments belonging to a larger group of polyphenolic compounds [6,7]. Flavanols are the main class of flavonoids found in tea. Although the total flavonoid content in green and black tea is similar, their chemical structures vary. The main reason for this is that the additional fermentation in black tea manufacturing facilitates the oxidation process and leads to the conversion of flavonoids (catechin) found in green tea into more complex varieties, mainly thearubigins and theaflavins [8, 9]. In green tea, catechin comprises 80–90% of the total flavonoids, whereas in black tea it accounts for 20–30% of the total flavonoids [10]. The constituent components and their respective quantities in green and black tea are presented in Table 15.1.

Green and black teas are associated with numerous health benefits. The main factor responsible for the effective therapeutic potential of both teas is attributed to catechins and theaflavins. The catechins and theaflavins vary in their structures and properties [11]. Among cate-

TABLE 15.1. Principal Components of Green and Black Tea.

Compounds	Black Tea (% weight of extract solids)	Green Tea (% weight of extract solids)
Catechins	3–10	30–42
Theaflavins	3–6	
Thearubigens	12–18	
Flavonols	6–8	5–10
Phenolic acids, depsides, and other Organic acids	10–12	6–8
Amino Acids	13–15	8–12
Methylxanthines	8–11	7–9
Carbohydrates	15	10–15
Protein	1	6–8
Mineral water	10	6–8
Volatiles	< 0.1	0.02

chins, the medicinal values are usually attributed to the epicatechins, rather than catechins shown in Figure 15.1. In trials with animals and humans, catechin and theaflavins showed health benefits, specifically in reactive oxygen species (ROS) scavenging, reducing inflammation, enhancing heart function, blood purification, lowering body temperature, strengthening teeth and bones, boosting the immune system, and slowing aging [4,10,11]. These activities of tea constituents are dependent on various factors, including metal-reducing potential, chelating behavior, pH, solubility characteristics, and bioavailability and stability in tissues and cells [12,13]. At present, the mechanisms of a number of tea activities have yet to be elucidated, and the exact structural features of certain theaflavins remain to be determined. The results of various studies showing the structural features and therapeutic potentials of green and black teas are summarized.

15.2. CHEMISTRY AND STABILITY

15.2.1. Chemistry of Tea Compounds

The chemical composition of tea leaves is well known. As mentioned, the origin of both green and black tea is the same and they have approximately similar chemical components. Table 15.1 presents a comparative quantitative analysis of chemical compounds contained in green and black tea. The main constituents of tea leaves are polyphenols, polysaccharides, proteins, chlorophyll, and alkaloids [14,15]. Among these, the polyphenols are considered to be the major active constituents of tea and are well recognized as tea catechins that make up approximately 25–35% of the dry weight of green tea leaves [16–18]. Catechins primarily belong to the flavonoid family, consisting of two benzene rings (A and B) with a central dihydropyran heterocycle (C ring). The structure of some basic catechins is shown in Figure 15.1. Rings A and B are identical to resorcinol and catechol moieties, respectively. It can be seen that carbon 2 and 3 of the catechin molecule possess chiral centers that govern n^2 (4) diastereoisomers, with two each for the *cis* and *trans* configurations. Catechin is the name given to the *trans* isomers, whereas *cis* isomers are known as epicatechin. The most important of the catechins are epigallo catechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) [4]. The antioxidant activities of the tea polyphenols are mainly attributed to the chemical structures and functional groups attached at

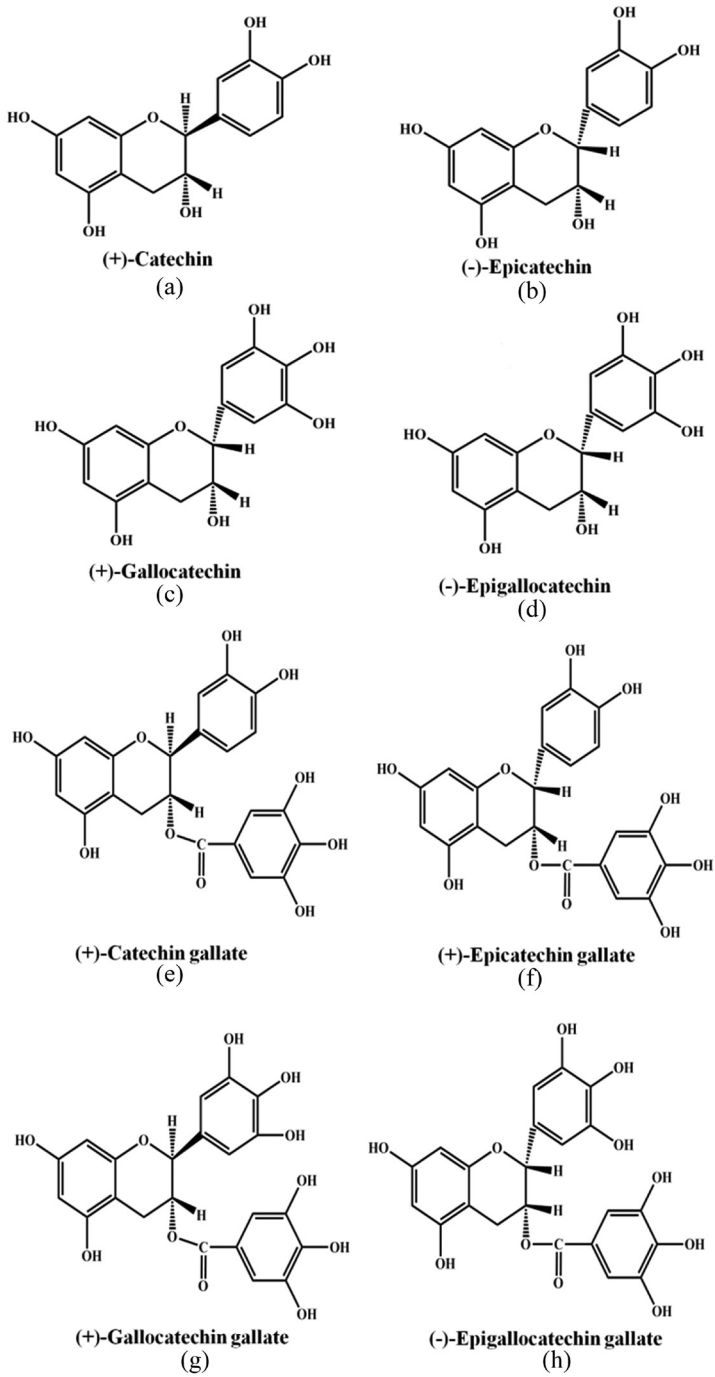

FIGURE 15.1. *Chemical structure of green tea catechins. The eight different types of catechins are (a) (+)-catechin, (b) EC, (c) GC, (d) EGC, (e) CG, (f) ECG, (g) GCG, (h) EGCG.*

various positions, including a di- or tri- hydroxyl group in the B-ring, an OH group at 5th and 7th carbon of the A ring, and a gallate group located at the 3 position of the C-ring [19]. The attachment of a gallate group governs additional catechins. All of these are shown in Figure 15.1.

As the manufacturing processes of green and black tea are very different, the types of polyphenols contained by both types of tea are also different. The major chemical changes in the black tea constituents take place during its extended fermentation process. It has been suggested that during the fermentation process, the catechins undergo phenolic oxidative coupling reactions and are converted to o-quinones. Further, the continued oxidation process results in the development of polymeric compounds, including polyphenols, theaflavins, and thearubigins. It has been suggested that theoflavins are the coupling oxidation products of EC and EGC [20]. The simplified oxidative mechanism of theaflavin production is shown in Figure 15.2. Whereas, Figure 15.3 illustrates the structures of four main theaflavins identified from black tea. These include theaflavin, theaflavin-3-gallate, theaflavin-30-gallate, and theaflavin- 3,30-digallate [11] .

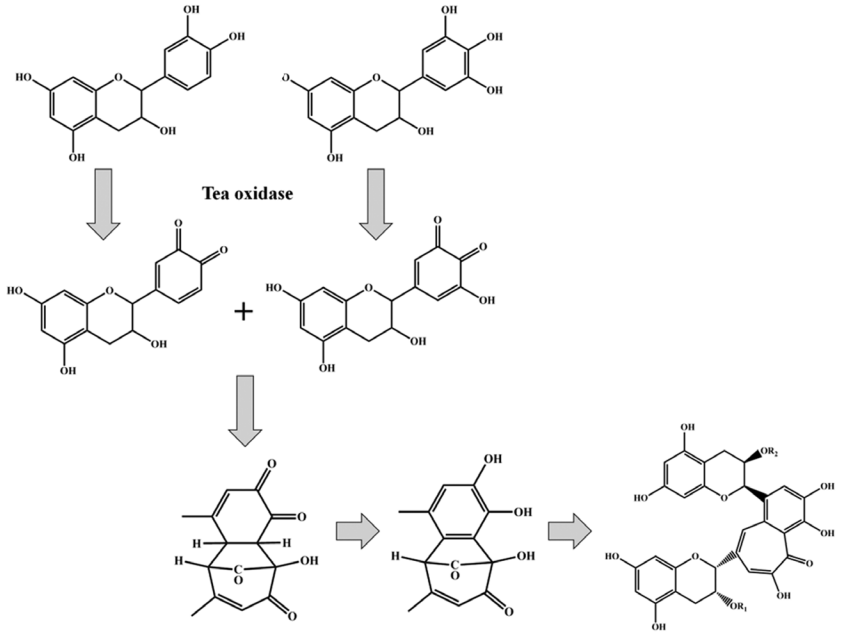

FIGURE 15.2. *A proposed mechanism of theaflavins formation from catechin oxidation.*

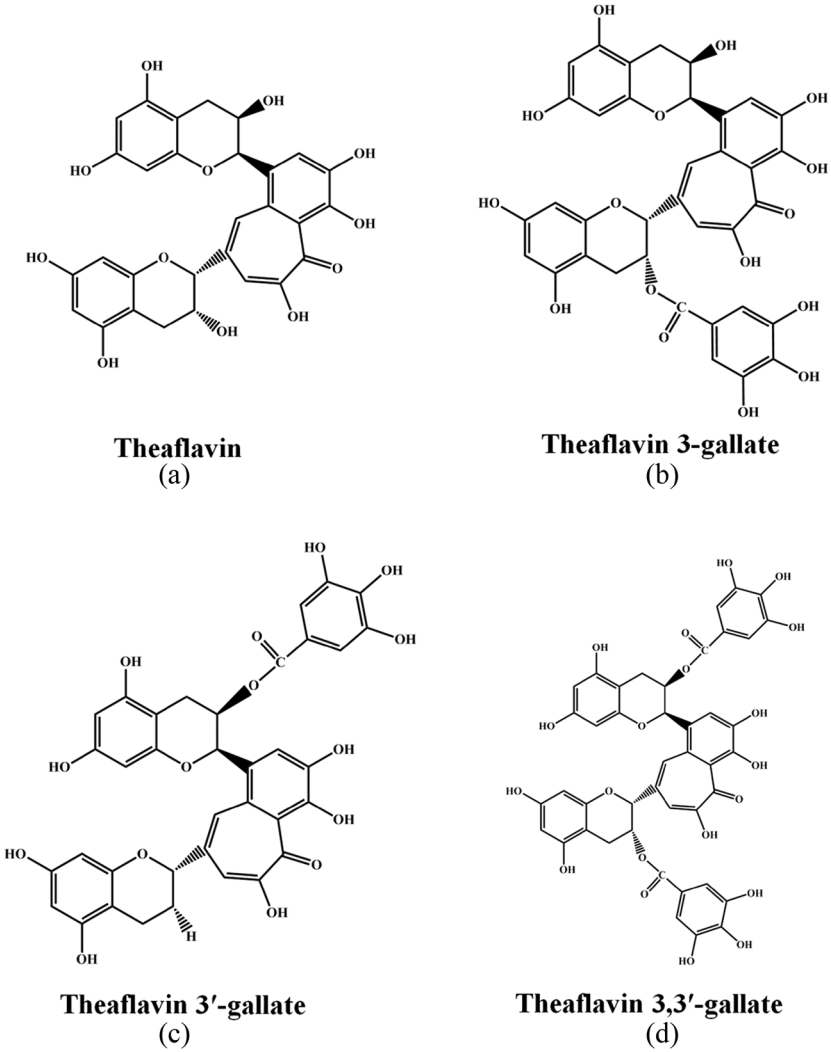

FIGURE 15.3. *Chemical structure of the four major black tea theaflavins. These include (a) theaflavins (TF1), (b) Theaflavin-3-gallate (TF2a), (c) Theaflavin-3′-gallate (TF2b) and (d) Theaflavin-3,3′-gallate.*

15.2.2. Stability of Tea Compounds

The stability of tea catechins is highly dependent on pH and temperature. Generally, the catechins are found to be unstable in neutral and alkaline pH environments, and show good stability under acidic conditions. A variety of chemical changes occur in catechins, mainly isom-

erization (epimerization), oxidation, decomposition to small molecules, and polymerization to oligomers or polymers under various conditions. It has commonly been observed that the color of tea changes from green (green tea) to brown upon boiling. The reason for this change is the degradation of molecules.

15.2.2.1. Effect of pH on the Stability of Tea Catechins

pH is very critical factor affecting the stability of catechins. In fact, catechins are extremely stable in acidic solutions (pH < 4), whereas in neutral or alkaline solutions (pH > 6), they are highly unstable [21,22]. It has been observed that green tea catechins were reduced to less than 10% when kept in a Krebs-Ringer buffer at pH 4, whereas they remained almost unchanged in water, and only a 15% reduction was observed after boiling for 7 hours [21,22]. The complete degradation of EGCG and EGC were reported in sodium phosphate buffer at pH 7.4 for 3 hours, whereas the ECG decreased by 20% and (–)-EC remained unchanged under the same conditions. Although the acidic conditions markedly increase the stability of catechins, the effect differs with the nature of the acids. The stability of green tea catechins was significantly increased with the addition of ascorbic acid, while citric acid did not provide any positive effect on the stability. Under alkaline conditions, the dimerized products of EGCG are converted to theasinensin (A and D rotational isomers) and P-2. Figure 15.2 illustrates the formation of products from EGCG. The proposed mechanism illustrates that EGCG was first dehydrogenated and then decarboxylated under oxidative conditions in an alkaline medium [23,24].

The capacity of various catechins for variation differs in similar environments. Among the four catechins examined in alkaline solutions, EGCG and EGC were equally unstable whereas EC and ECG were relatively stable. The exact reason for the observed diverse stability is yet to be clarified; however, the existence of an additional OH group at position 5 in EGCG is considered to be one of the factors. The three adjacent OH groups in EGCG and EGC were more susceptible to devastation than the two OH groups in EC and ECG [21]. Further studies have also shown the higher semiquinone free radical formation susceptibilities of ECG in alkaline media [25].

In addition to degradation or polymerization, the catechin (EC, ECG, EBC, and EGCG) molecules also exhibit small amounts of epimers produced from epimerization. The rate of epimerization varies under cer-

tain conditions that ultimately affect the relative amounts of the epimers. The rate of epimerization is also largely dependent on temperature, pH, buffer type, and concentrations of metal ions. The rate of epimerization was much higher at pH 6 than the rate in distilled water [26].

15.2.2.2. Effect of Temperature on the Stability of Tea Catechins

The stability of tea catechins is adversely affected at higher temperatures. As mentioned earlier, the color of green tea turns brown at high temperatures; an effect mainly attributed to the thermal variations in the chemical structure of catechins. The most predominant change upon heating is the conversion to isometric forms through epimerization. An important study demonstrated that epimerization of catechin was achieved when tea is subjected to a temperature of 40°C for an extended period of time [27]. The study clarified that not only temperature, but also time, are involved in the epimerization process. An example of the epimerization process is the conversion of epigallocatechin gallate to its epimer component gallocatechin gallate. It is also worth mentioning that the thermal process sometimes causes both epimerization and degradation to occur simultaneously [28]. It has been reported that epimerization of the four tea catechins, (–)-EC, ECG, EGC, EGCG, and 3″-*O*-methyl- EGCG, increased with increasing time at 90°C [29].

15.2.3. Effects of Oxygen Concentration and Metal Ions on the Stability of Tea Catechins

In addition to conditions (pH and temperature), the stability of tea catechins is also influenced by oxygen concentration and the presence of free radicals and metal ions. The catechins are oxidized more efficiently in the presence of higher oxygen levels and at lower antioxidant concentrations [30]. It is more appropriate to say that oxygen concentration is the main factor that facilitates the degradation of catechins, even at a suitable temperature and pH. In an N2 atmosphere (low O_2 concentration) at 37°C and pH 7.4, EGCG showed only a 5% reduction after 6 hours. This small reduction in EGCG concentration was also attributed to epimerization because no degradation product (dimers) was detected. In contrast, under normal conditions (O_2 atmosphere), an EGCG aqueous solution drastically degraded (90%) in 2 hours and completely diminished after 6 hours (no EGCG was left after 6 hours).

Metal ions are very reactive species. They react with catechins to

CHAPTER 22

Bioactivity, Bioavailability, and Human Health Effects of Berries' Bioactive Compounds

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22.1. BIOACTIVE COMPOUNDS RESPONSIBLE FOR FUNCTIONAL PROPERTIES OF BERRIES

Berry fruits, such as bilberry (*Vaccinium myrtillus*), blackberry (*Rubus fruticosus*), blackcurrant (*Ribes nigrum*), blueberry (*Vaccinium corymbosum*), chokeberry (*Aronia melanocarpa*), cranberry (*Vaccinium macrocarpon*), grape (*Vitis vinifera*), raspberry (*Rubus idaeus*), and strawberry (*Fragaria x ananassa*) are a particularly rich source of antioxidants. Those compounds are mainly represented by vitamin C and polyphenols such as anthocyanins, phenolic acids, flavonols, and tannins. They are known as natural antioxidants, and due to their high concentration and qualitative diversity, berry fruits are increasingly often referred to as natural functional foods. These findings have been confirmed in the research of Häkkinen and Törrönen (2000), Wang and Lin (2000), and Taruscio *et al.* (2004). As demonstrated by clinical research, the bioavailability of those naturally occurring compounds significantly exceeds the health benefits carried by their corresponding supplements in pharmaceutical form (Wang *et al.* 1996). Due to climatic conditions, fresh berries are generally available several months a year, while some

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of the harvested fruit is processed to juice, fruit beverages, frozen products, wine, jam, marmalade, and jelly (Da Silva *et al.* 2007).

Epidemiological studies indicate that persons who consume a diet rich in fruits, vegetables, and whole grains have a reduced risk of chronic diseases (Rui 2013). Fruits and vegetables are major sources of phytochemicals. Phytocemicals are defined as bioactive non-nutrient plant chemicals in fruits, vegetables, grains, and other plant foods that may provide desirable health benefits beyond basic nutrition to reduce the risk of major chronic diseases (Liu 2004). Among the fruits with this type of phytochemicals or bioactive compounds are berries. Commercially, the most important berries include members of the genus *Vaccinium* (blueberry, lingonberry, cranberry, bilberry), *Rubus* (blackberry, black raspberry, red raspberry, arctic raspberry/bramble, cloudberry), *Fragaria* (strawberry), and *Sambucus* (elderberry, red elderberry), and are usually consumed in fresh and in processed forms in the human diet (Stoner *et al.* 2008). The bioactive phytochemicals in berries fall into several structural and chemical classes including phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (anthocyanins, flavanols, flavonols), condensed tannins (proanthocyanins), hydrolyzable tannins (ellagitannins and gallotannins), stilbenoids, lignans, triterpenes, and sterols (Seeram 2006; Tulipani *et al.* 2008). In addition to the bioactives, berries contain many other constituensts including vitamins, such as A, C, E, and folic acid, and minerals, such as calcium, selenium, and zinc (Kresty *et al.* 2001). Berries contain high levels of a diverse range of phytochemicals, most of which are phenolic molecules, mainly anthocyanins and ellagitannins that, collectively, are responsible for much of their antioxidant activity (Connor *et al.* 2002; Wada *et al.* 2002; Cerda *et al.* 2005; Aaby *et al.* 2005), well as its color and flavor (anthocyanins and tannins, respectively). Studies reported that instrumental and sensory qualities of berries are affected by many factors such as cultivars, geographic region, storage conditions, ripeness, climate, and others may affect the concentration of phenolic compounds and in the antioxidant capacity (Castrejon *et al.* 2008; Seeram 2008; Zhang *et al.* 2008)

Berry fruits are good source of phenolics. Phenolics are defined as compounds possessing one or more aromatic rings with one or more hydroxyl groups in the estructures. Generally categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Seeram 2006). Phenolic compounds are secondary metabolism ubiquitous in all higher plants. These compounds have numerous defense functions in plants,

e.g., they act as defense mechanisms against pathogens, parasites, predators, and UV irradiation, and also contribute to the color of plants. They are often induced as a response to various stress conditions, e.g., light, temperature, humidity; and internal factors including genetic differences, nutrients, hormones, etc., contribute to their synthesis (Strack 1997). Phenolics occur in plant tissues as simple substituted phenols, mainly as glycosides, or as complex polymerized molecules with high molecular weights. The concentration of phenolic compounds varies between each species and varieties of berries, and is also affected by factors such as climate, geographic region, storage conditions, etc. (Kalt *et al.* 1999; Seeram 2008; Battino *et al.* 2009; Paredes-Lopez *et al.* 2010; Kim *et al.* 2013).

The chemistry of berry phenolics directly influences their bioavailability, metabolism, and biological effects in vivo (Manach *et al.* 2004, 2005). However, in general, the berries contain the following compounds: flavonoids such as anthocyanidins (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, malvidin), flavonols (quercetin, myricetin, kaempferol), flavonols (catechines) ((+)-catechin, (–)-epicatechin, gallocatechin, epigallocatechin), phenolic acids such as cinnamic acids (caffeic acid, p-coumaric acid, ferulic acid), benzoic acids (protocatchuic acid, p-hydroxybenzoic acid, gallic acid), lignans such as secoisolariciresinol, and complex phenolic polymers (polymeric tannins) such as ellagitannins (casuarictin) and proanthocyanidins (procyanidin trimer [flavonols]) (Seeram 2006; Paredes-Lopez *et al.* 2010).

Anthocyanins are one of the dominant groups of flavonoids in berries (up to 2,000–5,000 mg kg^{-1} fresh weight [FW]) (Määttä *et al.* 2001). They are good absorbers of visible light, comprise a large group of water-soluble pigments, and are responsible for the characteristic orange/red/blue colors of berries such as strawberries, raspberries, blueberries, and red and black currants. Their concentration is usually higher in the epidermis and in the tissue directly under the skin compared to the central part of the fruit (Paredes-López *et al.* 2010; Slatnar *et al.* 2012). For example, anthocyanins represent 44% of phenolic compounds present in strawberries. Anthocyanins comprise aglycones, anthocyanidins, and their glycosides anthocyanins (Viskelis *et al.* 2009). In Berry fruits, anthocyanins are found in the form of mono-, di-, or triglycosides, where glycoside residues are usually substituted at C3, or less frequently, at C5 or C7. The most prevalent sugars are glucose, galactose, rhamnose, arabinose, rutinose, sambubiose, and sophorose (Battino *et al.* 2009). Anthocyanin glycoside residues are often acylated by acids: P-cou-

maric acid, caffeic acid, ferulic acid, and by p-hydroxybenzoic acid, malonic acid or acetic acid (Viskelis *et al.* 2009). Caffeic and ferulic acids are the most common phenolic acids in berries, and they are rarely found free; in general they are esterified with other molecules as carbohydrates and organic acids. The most common esters of hydroxycinnamic acids are chlorogenic acid, which is an ester between caffeic acid and quinic acid, and is a commonly occurring compound in many berries. For example, chokeberry is a good source of hydroxycinnamic acid derivatives. They are represented by caffeic acid derivatives, chlorogenic acid (301.85 mg/100 g dry weight), and neochlorogenic acid (290.81 mg/100 g dry weight) (Lohachoompol *et al.* 2008). In cranberry fruit, the hydroxycinnamate esters are present in quantities averaging about 15 mg/100 g of fresh fruit (Pappas and Schaich 2009).

On the other hand, flavonoids and phenolic acids form the building blocks for polymeric tannins, which can be classified into hydrolysable and condensed tannins. Hydrolysable tannins are either gallotannins or ellagitannins, are less frequently encountered, and have been found in strawberries, raspberries, and blackberries (Holt *et al.* 2008). Berries, especially those of *Rosaceae* family genus *Rubus* (red raspberry, arctic bramble, and cloudberry), are rich in ellagitannins (Häkkinen *et al.* 200; Mullen *et al.* 2002). These berries and strawberries produce only ellagitannins based on stable glucose conformation. In addition to pentagalloylglucose, these berries contain dimeric or polymeric ellagitannins with amounts of monomers (Haslam 1989). Some berries, such as cranberry, also contain condensed tannins, called proanthocyanidins. Small quantities of tannins were found in honeyberry and blackberry. Lingonberry, strawberry, and cranberry are examples of berries rich in diphenolic compounds called lignans (10–15 mg kg^{-1} dry weight) (Mazur *et al.* 2000). Some other compounds found in berries is resveratrol (stilbenes), the compound found in grapes mainly, however also found small amounts of trans-resveratrol in bilberry (6.78 μg/g), cowberry (30 μg/g), redcurrant (15.72 μg/g), cranberry (19.29 μg/g), and strawberry (3.57 μg/g) (Ehala *et al.* 2005). The carotenoids are other compounds present in berries. Cloudberry, blueberry, cranberry, and cowberry contains 2,840, 2,140, 200, and 140 μg/100 g dry weight, respectively. These berries also contain β-carotene but this pigment prevailed greatly in cloudberry (83% of total carotenoids content). Lutein was the major carotenoid in blueberry (71%). Cranberry contains β-carotene (28%), lutein (23%), and neoxanthin (20%) (Lashmanova *et al.* 2012). On the other hand, chokeberry also of β-carotene and lutein contains lycopene,

β-cryptoxanthin, ζ-carotene, 5,6-epoxylutein, transviolaxanthin, cis-violaxanthin, and neoxanthin (Lashbrooke *et al.* 2010).

The presented characteristics of various berry fruit species point to vast differences in the type of their bioactive compounds. Such differences are observed with regard to both the content and the qualitative composition of those compounds. The most significant health benefits are ascribed to phenolic compounds and vitamin C. Owing to the rich and diversified compositions of bioactive compounds and their health-promoting properties which result mostly from their antioxidant activity, berry fruits are widely recognized as natural functional products (Szajdek and Borowska 2008).

22.2. ANTIMICROBIAL ACTIVITY OF BERRY BIOACTIVE COMPOUNDS

The development of food products or ingredients with specific health promoting benefits (nutraceuticals or functional foods) is currently the fastest growing and most consumer-driven segment of the food industry (Hussein *et al.* 2011). On the other hand, food-borne illnesses, the spread of antibiotic-resistant pathogens, and concerns regarding safety of synthetic antimicrobial agents have increased consumer demand for the use of plant extracts as natural antimicrobials and antioxidants in foods (Al-Zoreky 2009; Staszewski *et al.* 2011). Nature offers many different types of antimicrobial compounds that play an important role in the natural defense of all kinds of living organisms (Rodriguez Vaquero, *et al.* 2007). Plant extracts containing flavonols, other phenolic compounds, and organic acids are potent antioxidants and some of them have shown additionally good antimicrobial activity, which makes their possible use in food systems reasonable (Choi *et al.* 2006; Kalogeropoulos *et al.* 2009).

Fruits and berries contain a variety of phenolic compounds located in plant tissues, often in the surface layer of the plant, fruit, or berry, which is in connection to their main natural function, to protect the plant against environmental stress and pathogens. Berries are rich in phenolic compounds, which are classified into four main groups: flavonoids, phenolic acids, lignans, and polymeric tannins (Nohynek *et al.* 2006). Flavonoid anthocyanins are common in bilberry, black and red currant, chokeberry, strawberry, and raspberry, in which they appear as colored substances (Määttä-Riihinen *et al.* 2004). Ellagitannins are complex phenolic polymers, which are the main phenolic compound in red raspberry and cloudberry.

Strawberries also contain ellagitannins, although the amounts are lower (Aaby *et al.* 2005). The most common phenolic acids present in berries are derivatives of either hydroxycinnamic acids or hydroxybenzoic acids. Lingonberry, strawberry, and cranberry are examples of berries containing lignans (Nohynek *et al.* 2006).

Probiotic bacteria are widely used in functional foods. Therefore, there is an interest in probiotic bacteria such as *Lactobacillus acidophilus* as well as *Bifidobacterium spp.* together with plant derived compounds, especially in their application in food technology, for their positive impact on human health (Pascual-Teresa *et al.* 2010).

Antimicrobial activity of plant phenolics has been intensively studied, and, in addition to controlling invasion and growth of plant pathogens, their activity against human pathogens has been investigated to characterize and develop new healthy food ingredients, medical compounds, and pharmaceuticals (Chung *et al.* 1998). Berries are an important part of the Nordic diet, but they have also been used as natural antimicrobial pharmaceuticals. Bilberry has been used, for example, for gastrointestinal (GI) disorders, and cranberry has been a well-known treatment in urinary infections. For example, cranberry has been reported to control the growth of *Listeria monocytogenes* (Puupponen-Pimiä *et al.* 2005; Nohynek *et al.* 2006) and to possess compounds suppressing adhesion and growth of *Helicobacter pylori* (Vattem *et al.* 2005) and bacteria causing urinary tract infections (Nohynek *et al.* 2006).

On the other hand, certain berries rich in tannins have been found to increase bacterial infections. Two different types of polymeric tannins in these berries protect against pathogenic bacteria. Presence of the A-type linkage of cranberry (*Vaccinium macrocarpon*) proanthocyanidins may enhance both in vitro and urinary bacterial antiadhesion activities (Nile and Park 2013). Additionally, other tannin-containing berries may contribute to this effect, as berry juices of a mixture of cranberries (*Vaccinium oxycoccus*) and lingonberries as well as cloudberry juice protect against urinary tract infection (Kontiokari *et al.* 2003).

Among the berries, cranberries, cloudberries, red raspberries, strawberries, and bilberries possess clear antimicrobial effects against human pathogens. Berry ellagitannins are strong antimicrobial agents acting as possible antiadherence compounds in preventing the colonization and infection of many pathogens (Puupponen-Pimia *et al.* 2005). The phenolic extract of cloudberry, which is comprised primarily of ellagitannins, has the strongest antimicrobial effect, followed by red raspberry and strawberry. *Salmonella spp.*, *Staphylococcus spp.*, *Helicobacter*

spp., and *Bacillus spp.* are the bacteria that are most sensitive to berry phenolics. Additionally, the growth of *Escherichia spp.*, *Clostridium spp.*, and *Campylobacter spp.*, but not the growth of *Lactobacillus spp.* and *Listeria spp.*, is inhibited by berry phenolics (Nile and Park 2013). Red raspberry phenolics and its ellagitannin fraction also have powerful antimicrobial properties against the growth of human colonial pathogens, *Klebsiella oxytoca* and *Proteus mirabilis* (Nurmi *et al.* 2009). Several mechanisms of action in the inhibition of bacteria are involved, such as destabilization of cytoplasmic membrane, permeabilization of plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, and deprivation of the substrates required for microbial growth (Kraft *et al.* 2008). The berry extracts inhibited the growth primarily of gram-negative bacteria but had no effect on gram-positive bacteria. However, there is very little information about the antimicrobial capacity of phenolics present in berries, except in cranberry. Extracts of the aerial parts of bearberry and lingonberry were active against the gram-negative bacteria *E. coli* and *P. vulgaris*. The activity is known to be due to the phenolic glycosides arbutin and metylarbutin.

Cloudberry, raspberry, and strawberry extracts were the strongest inhibitors of gram-negative bacteria, especially *Typhimurium spp.* (Chung 1998). Ellagic acid inhibits a range of pathogenic organisms including *Vibrio cholerae*, *Shigella dysenteriae*, and *Campylobacter spp*. It can be hypothesized that ellagitannins could be one of the components in cloudberries, raspberries, and strawberries causing the inhibition against *Salmonella*. The antimicrobial effects of berry extracts against gram-negative bacteria decreased in the following order: cloudberry > raspberry > strawberry > lingonberry > blueberry > cranberry > sea buckthorn berry > blackcurrant (Nile and Park 2013).

22.3. EFFECT OF BERRIES CONSUMPTION AGAINST CHRONIC DISEASES DEVELOPMENT

Berries are known as being a good source of vitamin C, dietary fiber, and minerals, and they contain high amounts of phenolic compounds, including anthocyanins, chlorogenic acids, flavonols, and procyanidins, resulting in high antioxidant activity that provides health benefits, and conferring on berries the title of a functional food (Huang *et al.* 2012). Berry extracts, rich in phenolic compounds and anthocyanins, have a range of biological effects which prevent or reduce the incidence of

chronic disorders, including cancer, cardiovascular disease, diabetes, inflammation, cancer, Parkinson´s and Alzheimer´s disease, and other pathologies (Knekt *et al.* 1996, 1997; Wang *et al.* 2000; Krikorian *et al.* 2010) These diseases are caused, in part, by the conversion of cellular macromolecules to specific reactive oxygen species (ROS) during normal cellular metabolism (Weisburger 1999). ROS and free radicals are produced in an extensive range of physiological processes. Oxidative stress is an imbalance between the production of ROS and antioxidant defense, and may lead to oxidative damage. This biological condition may result from an insufficiency of antioxidant defense mechanisms, intense production of ROS, and excessive activation of ROS systems, which are implicated in aging and in the pathology of many chronic disorders (Liu and Hotchkiss 1995; Winterbourn 2008). In order to confront oxidative stress, human bodies have developed mechanisms for maintaining redox homeostasis. These mechanisms include the nonenzymatic and enzymatic antioxidant defenses produced in the body, that is endogenous, and others supplied by the diet namely exogenous ones. The exogenous antioxidants include phenolic acids, flavonoids, stilbenes, and tannins (Seeram 2008; Winterbourn 2008). Phenolic compounds exhibit many biologically significant mechanisms of action, such as scavenging or detoxification of ROS, blocking ROS production, impacting cell cycle, suppression of tumors, modulation of signal transduction, apoptosis, detoxifying enzymes, and metabolism (Liu 2004; Hand *et al.* 2007). Berries contain high levels of natural polyphenol components that act as potent antioxidants. Berry extracts, rich in polyphenols, have a range of biological effects that can have beneficial outcomes on human health.

Studies indicate that extracts of berries cardioprotective effect (Whitson *et al.* 2004). For example, mechanisms for the prevention of atherosclerosis by dietary antioxidants in fruits have been proposed. In the LDL oxidation hypothesis, oxidized LDL cholesterol by free radicals has been suggested as the atherogenic factor that contributes to cardiovascular disease (CVD) (Berliner *et al.* 1997; Witztum and Berliner 1998). When circulating LDLs are present at high levels in blood, they infiltrate the artery wall and increase intimal LDL, which can then be oxidized by free radicals. This oxidized LDL, in the intima is more atherogenic than native LDL and serves as a chemotactic factor in the recruitment of circulating monocytes and macrophages. Dietary phytochemicals served as antioxidants that are incorporated into LDL are themselves oxidized when the LDL is exposed to free radicals; this

occurs before any extensive oxidation of the sterol or polyunsaturated fatty acids can occur (Sanchez-Moreno *et al.* 2000). Therefore, they are very effective inhibitors of low density lipoprotein oxidation, a key step in the development of atherosclerosis as described in the study by Heinonen *et al.* (1998) from inhibited human LDL and liposome oxidation for phenolic extracts of berries (blackberries, red raspberries, sweet cherries, blueberries, and strawberries), also have beneficial effects on platelet aggregation. At low levels, they provide protection of nitric oxide levels in arterial systems. Nitric oxide is crucial for the maintenance of flexible blood vessels and thereby important in controlling blood pressure. The same author indicates that the antioxidant activity of a Berry extract in the LDL oxidation was related to the presence of anthocyanins, but the activity in the liposome oxidation was correlated with the amount of hydroxycinnamates. In complex lipid systems where several different antioxidant and pro-oxidant actions occur simultaneously, it is obviously more difficult to observe the effect of a single factor than in simplified radical scavenging models. In lipid oxidation models, peroxyl radical scavenging and metal inactivation properties are very important mechanistic factors, but the polarity of the compound and the physical state of the lipid system also affect the behavior of antioxidants. In addition, synergism, that is, the ability of antioxidant compounds to reinforce each other, can have a significant effect on the antioxidant response (Frankel 1998).

In addition, dietary phytochemicals can lower C-reactive protein (CRP) dramatically. C-reactive protein, a marker of systemic inflammation, has been reported to be a stronger predictor of CVD (Ridker *et al.* 2002). An Iranian study in Tehran found that both fruits and vegetables were inversely associated with plasma CRP concentrations (Esmaillzadeh *et al.* 2006), as did a 2004 study in Massachusetts (Gao *et al.* 2004). Therefore, the anti-inflammatory activity of phytochemicals may play an important role in the prevention of CVD. In addition, dietary phytochemicals have been shown to have roles in the regulation of prostaglandin synthesis, reduction of platelet aggregation, regulation of cholesterol synthesis and absorption, and reduction of blood pressure.

On the other hand, berry bioactive have many roles in cancer prevention and certain berries are considerably more effective than others. For example, the most active components in raspberry were the ellagitannins, but these components break down readily and the resultant products, including ellagic acid, may be the actual active components (Ross *et al.* 2007). Roles in cancer prevention include protection

against oxidative DNA damage by the scavenging of ROS, inhibition of the formation of carcinogen-induced DNA adducts, enhancement of DNA repair, inhibition of carcinogen-induced tumorigenesis in animals, and modulation of signaling pathways involved with cellular proliferation, inflammation, angiogenesis, modulate cell cycle arrest, and induce apoptosis (programmed cell death). A study by Seeram *et al.* (2006), showed that blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit the growth of human oral (KB and CAL27), breast (MCF-7), colon (HT29, HCt 116), and prostate (LNCaP) cancer cell lines in a dose-dependent manner. The extent of inhibition of cell proliferation was found to vary markedly with both the type of Berry extract and the specific cell line studied. The effects of the different berry extract on cell apoptosis was assessed using HT29 cell that were treated with 200 μg/ml of each Berry extract. Strongest inhibition of cell growth was observed for the raspberry, lowbush blueberry, and cranberry juices. The authors reported that the inhibition of proliferation by the berry juices was independent of caspase-dependent apoptosis but appeared to involve cell-cycle arrest, as evidenced by down-regulation of the expression of cyclin dependent kinases, cdk4, cdk6, cyclin D1, and cyclin D3. Some of the berry juices also significantly inhibited the tumor necrosis factor-induced activation of the COX-2 enzyme expression and activation of the transcription factor, nuclear factor kappa B (NF-κB). The authors concluded that different Berry fruits might act through different mechanisms in their cancer preventive ability (Boivin *et al.*, 2007). Ding *et al.* (2006) investigated the effects of cyanidin-3-*O*-glucoside, isolated from blackberries, on gene expression in JB-6-cells. Cyanidin-3-*O*-glucoside pretreatment led to dose-dependent decrease in the expression of cyclooxygenase-2 (COX-2) and activities of AP-1, NF-κB, and tumor necrosis factor (TNF)α, when the cells were treated with 12-*O*-tetradecanolyphorbol-13-acetate or ultraviolet. Finally, and anthocyanin-rich preparation from berries (wild blueberry, bilberry, cranberry, elderberry, raspberry, and strawberry), was found to reduce vascular endothelial growth factor (VEGF) expression in a spontaneously immortalized human keratinocyte cell line (HaCaT) and inhibit endothelial tube formation in a Matrigel assay (Bagchi *et al.* 2004).

A study recently investigated whether the regulations of apoptosis and the phase-II enzymes glutathione-S-transferase (GST) and quinone reductase (QR) are potential mechanisms through which blueberry may prevent cancer. Srivastava *et al.* (2007) showed that anthocyanin-enriched fractions purified from the blueberries induced apoptosis of

CHAPTER 27

Emerging Sources for Marine Nutraceuticals

E. APOSTOLIDIS and C.M. LEE

27.1. INTRODUCTION: GLOBAL PERSPECTIVE IN MARINE NUTRACEUTICALS

Jacques Cousteau, who is considered a father of ocean exploration, once said, "The future of nutrition is found in the oceans." While the majority of nutraceutical products in the current market are of botanical origin, marine-based nutraceuticals are gaining attention due to their unique features which are not found in the terrestrial-based resources. In recent years, a series of promising new marine nutraceutical products have been introduced to the nutraceutical and functional foods markets. Currently, functional foods are one of the major consumer trends in the food industry. Consumers continue to seek multiple ways to enhance their health to prevent diseases, and to promote healthy aging by paying more attention to what they are eating and how it benefits their health. Today, consumers are more concerned about their weight, cardio-health, digestive-health, and immunity than ever before. Since health is a major concern of consumers, manufacturers are finding new ways to incorporate natural and innovative ingredients into food products for health benefits. The sales of functional foods and beverages have been rising with a constant 8% per year since 2003. In 2006, the market of func-

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tional foods and beverages was $195 billion and is estimated to reach $195 billion by 2013. Functional foods are defined as "those foods that encompass potentially healthful products including any modified food or ingredient that may provide a health benefit beyond the traditional nutrients it contains." These ingredients vary and include antioxidants (vitamins and phenolic phytochemicals), omega-3 fatty acids, plant sterols, and many more. In recent years, a series of promising new marine nutraceutical products have been introduced to the nutraceutical and functional foods markets. This chapter presents an outline of emerging sources for marine nutraceutical development. The main sources and products of primary interest for emerging marine nutraceuticals include phospholipids bound omega-3 fatty acids, micro/macro algal nutrition supplements, fish proteins and peptides, and shellfish derived oligomolecules of chitin. Also discussed are emerging products, production methods, the ongoing R&D activities, and challenges in marine nutraceutical markets, along with future developments in marine nutraceutical industry and research.

27.2. PHOSPHOLIPID-BOUND OMEGA-3 FATTY ACIDS

Fish oils are a rich source of long-chain omega-3 polyunsaturated fatty acids (PUFA), which have attracted much attention in the recent years. Fish oil is produced from various sources of marine fish, i.e., anchovy, menhaden, herring, mackerel, salmon, cod liver, and mussel, and marketed in various forms, most commonly concentrated omega-3 oil in soft gel capsules and microencapsulated powder. Phospholipids of marine origin or phospholipid-bound omega-3 fatty acids are considered a good source of more bioavailable and more stable omega-3 fatty acids when compared to free omega-3 fatty acids. Currently, marine phospholipids are produced from krill and squid in commercial quantities.

27.2.1. Krill Derived Phospholipids

Krill, which means "whale food" in Norwegian, are eucarid crustaceans divided into two families. Krill occur in all oceans of the world. They are considered an important trophic connection—near the bottom of the food chain—because they feed on phytoplankton and to a lesser extent zooplankton, converting these into a form suitable for many larger animals for whom krill makes up the largest part of their

diet (Baker *et al.* 1990; Brinton 1962). The Bentheuphausiidae family consists solely of *Bentheuphausia amblyops* (Brinton 1962), a deep water krill species that differs from members of the Family Euphausiidae in that *Bentheuphausia amblyops* is not bioluminescent. The Euphausiidae family includes the other 89 known krill species, including one of the most common, *Euphausia superba* (Brinton 1962), which is the most frequent species associated with krill. Well-known species—mainly because they are subject to commercial krill fishery—include Antarctic krill (*Euphausia superba*), Pacific krill (*Euphausia pacifica*), and Northern krill (*Meganyctiphanes norvegica*) (FAO 2010). The two major krill fisheries are the Antarctic Ocean and the North Pacific Ocean along with the coasts of Japan and Canada (FAO 2010). While about 100,000 tons of krill are harvested annually, this represents only about 0.1% of the estimated existing population (Deutsch 2007). Whole krill are composed of 60–80% protein, 7–26% lipid, and 12–17% ash on a dry weight basis (Grantham 1977; Pierce *et al.* 1969). Krill oil extracted from Antarctic krill (*E. superba*) is rich in phospholipid-bound omega-3 fatty acids, mainly EPA and DHA. Krill oil also contains various potent antioxidants, including vitamins A and E, astaxanthin, and a novel flavonoid (similar to 6,8-di-c-glucosylluteolin but with two or more glucose molecules and one aglycone) (Bunea *et al.* 2004; Deutsch 2007; Sampalis *et al.* 2003). Krill oil for dietary commercial applications is extracted by a cold vacuum extraction process that protects the biomass from exposure to heat, light, or oxygen to prevent lipid oxidation and maintain the original nutrients of krill intact (Deutsch 2007).

27.2.2. Squid Byproduct Derived Phospholipids

Squid belongs to *marine cephalopods* of the *order* Teuthida, which comprises around 300 species (Clarke *et al.* 1996). Like all other cephalopods, squid has a distinct head, *bilateral symmetry*, a *mantle*, and eight *arms* arranged in pairs and two longer *tentacles* (Clarke *et al.* 1996). According to the *FAO*, the cephalopod catch for 2002 was 3,173,272 tons of which 2,189,206 tons, or 75.8%, were squid (FAO 2003). In the northeastern United States, approximately 19,100 tons of squid were landed annually since 1998 with steady landings projected (Cardin 2000; NOAA 2008). Due to the high demand for cleaned squid in the United States market, most of harvested squid is processed resulting in 40–50% of unutilized byproducts, causing a serious disposal

problem. According to Point Judith Fishermen's Co. (Narragansett, RI) and Sea Fresh USA (North Kingstown, RI), it is estimated that squid processing plants, which are located mainly in Rhode Island, New York, and New Jersey, generate in excess of 4,500 tons of processing byproduct annually. Squid processors pay to dispose of the squid processing byproduct at a rate of $65–$90/ ton (approximately $100,000/ year per processor). The squid processing byproduct consists mainly of head, fin, and viscera, along with unclaimed mantles and tentacles, and contains approximately 11–14% protein, 2–3% lipids (mostly phospholipids), with 11.6% EPA and 24.5% DHA omega-3 fatty acids in oil, 0.6–1.3% ash, and 84–85% moisture (Lian et al 2005). The lipid content of squid byproducts is around 2.5% (De Koning 1993; Lian *et al.* 2005) and 75% of total lipid is in the form of phospholipids (De Koning 1993; Lian *et al.* 2005).

27.3. HEALTH BENEFITS

27.3.1. Bioavailability: Nonphospholipid Bound versus Phospholipid Bound Omega-3 Fatty Acids

Omega-3 fatty acids are considered essential fatty acids since they are necessary for human health but the body can't make them and need to be delivered either through diet or dietary supplements. However, in order to have a physiological and health beneficial effect they need to be first absorbed through the intestinal wall and then from the cells of interest for the specific health benefit. Several studies indicate the beneficial role of phospholipids in enhancing the therapeutic efficacy of some molecules that have poor absorption. Since the 1980s, phospholipid complexes began to be a common method to increase bioavailability and absorption of several drugs (Ranade 1989). Silibinin, also known as silybin, is the major active constituent of silymarin, the mixture of *flavonolignans* extracted from *blessed milk thistle* (*Silybum marianum*) and is a molecule that has poor water solubility and bioavailability. It was observed that the silybin-phospholipid complex has significantly higher therapeutic efficacy over the pure molecule in protecting the liver and exerting antioxidant activities (Carini *et al.* 1992; Comoglio *et al.* 1995; Conti *et al.* 1992; Morazzoni *et al.* 1993; Yanyu *et al.* 2006). Quercetin is a *flavonol*, plant-derived flavonoid, used as a *nutritional supplement* that has been reported to have poor absorption (Hollman *et al.* 1996, 1997; Manach *et al.* 1996; Miyazawa *et al.*

1999; Scalbert and Williamson 2000). Recent studies have shown that queretin-phospholipid complex has better therapeutic efficacy than the compound alone in rat liver injury induced by carbon tetrachloride (Maiti *et al.* 2005) and enhanced absorption in rats (Azuma *et al.* 2002). Curcumin is a low molecular weight polyphenol derived from the rhizomes of turmeric (*Curcuma longa*) that has a vast array of beneficial pharmacological effects (Bush *et al.* 2001; Gescher *et al.* 2001; Ireson *et al.* 2001; Park *et al.* 2000; Perkins *et al.* 2002; Ruby *et al.* 1995; Shao *et al.* 2001; Sharma *et al.* 2001a; 2001b). Despite the promising biological effects of curcumin, poor bioavailability in both rodents and humans was reported by several researchers (Ammon *et al.* 1991; Ireson *et al.* 2001; Pan *et al.* 1999; Wahlström *et al.* 1978). Recent studies have shown that curcumin phospholipid-complex has better hepatoprotective activity (Maiti *et al.* 2007) and better bioavailability (Liu *et al.* 2006) than free curcumin.

Omega-3 fatty acids have been found to be readily absorbed (Nakamura and Nara 2004) in the small intestine once they are released from lipids, after being hydrolyzed by pancreatic enzymes (Lichtenstein and Jones 2001). However, research studies have shown that the association between phospholipids and long-chain omega-3 fatty acids highly facilitates the passage of fatty acid molecules through the intestinal wall, increasing bioavailability and ultimately improving the omega-3:omega-6 fatty acid ratio (Cansell *et al.* 2003; Werner *et al.* 2004), thus increasing the health beneficial efficacy of omega-3 fatty acids. Recent studies indicate that phospholipid-bound omega-3 fatty acids have better efficacy for premenstrual syndrome management (Sampalis *et al.* 2003) and hyperlipidemia management (Bunea *et al.* 2004) when compared to fish oil non phospholipid-bound omega-3 fatty acids. A recent clinical study compared the bioavailability of EPA and DHA between krill oil and menhanden oil (Maki *et al.* 2009). The daily quantity of EPA provided in the krill (216 mg) and menhanden oil (212 mg) in this study was comparable. However, the DHA present in krill oil (90 mg/d) was approximately half of that provided in the menhanden oil (178 mg/d). At the end of the treatment period (4 weeks), the mean plasma EPA concentration was slightly higher in the krill oil group when compared to menhanden oil group (377 versus 293 μmol/ L), whereas the mean plasma DHA concentrations were comparable (476 versus 478 μmol/ L) (Maki *et al.* 2009). These results suggest that phospholipid-bound EPA and DHA from krill oil could be better absorbed thank those from menhaden oil. Since the concept of phospholipid-bound

omega-3 fatty acids is emerging and taking into consideration their positive effect on nutraceutical compound bioavailability, more studies on the effect of the phospholipid complex on omega-3 fatty acid bioavailability are expected in the coming years.

27.3.2. Omega-3 Fatty Acids and Cardiovascular Disease

Cardiovascular disease (CVD) is an abnormal function of the heart or blood vessels. It can cause an increase in risk for heart attack, heart failure, sudden death, stroke, and cardiac rhythm problems, thus resulting in decreased quality of life and decreased life expectancy. The causes of cardiovascular disease range from structural defects to infection, inflammation, environment, and genetics. One in three American adults has some form of CVD. Heart disease and stroke are the most common cardiovascular diseases and are the first and third leading causes of death for both men and women in the United States (Mayo clinic 2010). CVD is the leading cause of deaths in the Western societies (Delorgeril 2001) and has been linked to the high fat intake, particularly saturated fats, common in Western diets (Dolecek and Granditis 1991). The association between omega-3 fatty acids and cardiovascular disease was first indicated in the 1950s following comparison studies between Greenland Inuits and Danish settlers of Greenland (Sinclair 1956). Both groups were consuming approximately the same amounts of fat (40% of caloric intake), however, the Greenland Inuits had lower incidence of heart disease, despite the higher intake of dietary cholesterol (Sinclair 1956). Later in the 1970s, the low mortality rate from coronary heart disease of Greenland Inuit, was reported to be linked to the high omega-3 fatty acid in the Inuit diet which consisted mainly of fish, seal, and whale (Dyerberg *et al.* 1975). There are many theories regarding the mechanisms of action of omega-3 fatty acids in cardiovascular disease. It is thought that they have a multifactorial mode of action including antiarrhythmic effects, antithrombotic effects, antiatherosclerotic effects, anti-inflammatory effects, improvement in endothelial function, lowering of blood pressure, and lowering of triglyceride concentrations (Albert *et al.* 2002; Harris 2007; 2006; Kang and Leaf 2000; Knapp 1997; Morris *et al.* 1993; Nair *et al.* 1997). In the recent years, clinical trials have assessed the effects of fish and fish oil on CVD prevention. The diet and reinfarction trial (DART) showed a 29% reduction of mortality rate among 2,033 men with a recent myocardial infarction, linked to the omega-3 fatty acid consumption (Burr *et al.* 1989). The Gruppo Italiano

per lo Studio della Sopravvivenza nell'Infarto Miocardico Prevenzione (GISSI-Prevenzione) trial that lasted for three-and-a-half years showed 30% reduction of risk of cardiovascular disease death and 45% reduction of sudden death incidence among 11,324 patients after myocardial infarction and was linked to omega-3 fatty acid consumption (Grupo Italianoperlo Studio 1999). In the Asian population, patients with a suspected myocardial infarction showed a significant reduction in mortality rate from coronary heart disease due to omega-3 fatty acid supplementation (Singh *et al.* 1997). A clinical trial compared the effect of krill oil derived phospholipid bound omega-3 fatty acids with fish oil and according to Bunea *et al.* (2004), krill oil was significantly more effective than fish oil for the reduction of glucose, triglycerides, and LDL levels. Another clinical study with 76 overweight and obese adults compared the effect of krill oil phospholipid-bound omega-3 fatty acids and menhanden oil and according to Maki *et al.* (2009), krill oil supplementation produced significant elevations in plasma levels of EPA and DHA. The above findings confirm the better potential of phospholipid-bound omega-3 fatty acids for cardiovascular disease prevention, mainly due to better bioavailability.

27.3.3. Omega-3 Fatty Acids and Inflammatory Disease

Inflammation is an important component of the early immunological responses, while inappropriate or dysfunctional immune responses underlie chronic inflammatory and autoimmune diseases. Onset of autoimmune and inflammatory diseases has been linked to omega-3 and omega-6 imbalance (James *et al.* 2000; Simopoulos 2002; Song 2008),with omega-3 fatty acids exerting anti-inflammatory properties (Shahidi 2007; Simopoulos 2002; Song 2008). Arachidonic acid, an omega-6 fatty acid, is the precursor of eicosoanoids that produce compounds that activate macrophages and induce inflammatory response (Simopoulos 2002; Song 2008). On the other hand, EPA, an omega-3 fatty acid, competes with arachidonic acid (Simopoulos 2002) and has been shown to inhibit certain immune functions, such as the production of ecosanoids and proinflammatory cytokines(Song 2008). Several scientific findings have indicated the positive effect of omega-3 fatty acids on inflammatory disease management (Shahidi 2007; Song 2008). These findings have shown that omega-3 fatty acids can reduce proinflammatory cytokine production (Din *et al.* 2004; Hulshof *et al.* 1999; Kamal-Eldin and Yanishlieva 2002; Newton and Snyder 1997;

Watanabe and Ackman 1974), manage bronchial inflammation (Holmer 1989), and arthritis (Shahidi 2007). In regards to phospholipid-bound omega-3 fatty acids, a clinical study with 90 patients has shown that krill oil inhibits inflammation and reduces arthritic symptoms within a short period of treatment (Deutsch 2007).

27.3.4. Omega-3 Fatty Acids and Brain Functions

All membranes, whether they are cell surface or form of an intracellular organelle, are composed of phospholipid bilayers. Membrane lipids provide flexibility and adaptable structure into which are inserted proteins and glycoproteins such as enzymes, transporter proteins, or receptors (Farquharson *et al.* 1995). More specific research has shown that phospholipids of the brain have an especially high content of the long chain omega-3 fatty acid, DHA, and that these phospholipids are involved in brain function (Amaducci *et al.* 1991; Hirata and Axelrod 1980; Salem and Niebylski 1995) The structural predominance of DHA in the brain suggests functional significance and according to Young and Conquer (2008), both DHA and EPA are linked with several aspects of neural function, including phospholipase A_2 (PLA_2) activity, inflammation, neurotransmission, membrane fluidity, oxidation, ion channel, enzyme regulation, and gene expression. It has been suggested that under pathological conditions, seen in neuropsychiatric disorders such as schizophrenia, an increase in the activity of PLA_2 can results in a decrease in neuronal membrane phospholipid biosynthesis and an increase in phospholipid breakdown (Horrobin 1998). Finnen and Lovel (1991) have shown that EPA can inhibit PLA_2 and according to Mellor *et al.* (1996), administration of EPA has shown considerable success in the treatment of schizophrenia and bipolar disorder. Modulation of neurotransmitters, such as serotonin and dopamine, has been shown to be a causative factor for unipolar and bipolar depression (Mundo *et al.* 2001; Ogilvie *et al.* 1996; Post *et al.* 1980). Several studies have shown that supplementation through diet of DHA and EPA contribute towards the increased concentrations of serotonin and dopamine (Austed *et al.* 2000; Chalon and Delion-Vancassel 1998; Zimmer *et al.* 2000,1999; Owens and Innis 1999). A clinical study on 70 patients diagnosed with premenstrual syndrome showed that supplementation of phospholipid containing krill oil reduced the emotional symptomatology that characterizes premenstrual syndrome (Sampalis *et al.* 2003). In this study, it was suggested that the effectiveness of krill oil could be based on po-

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