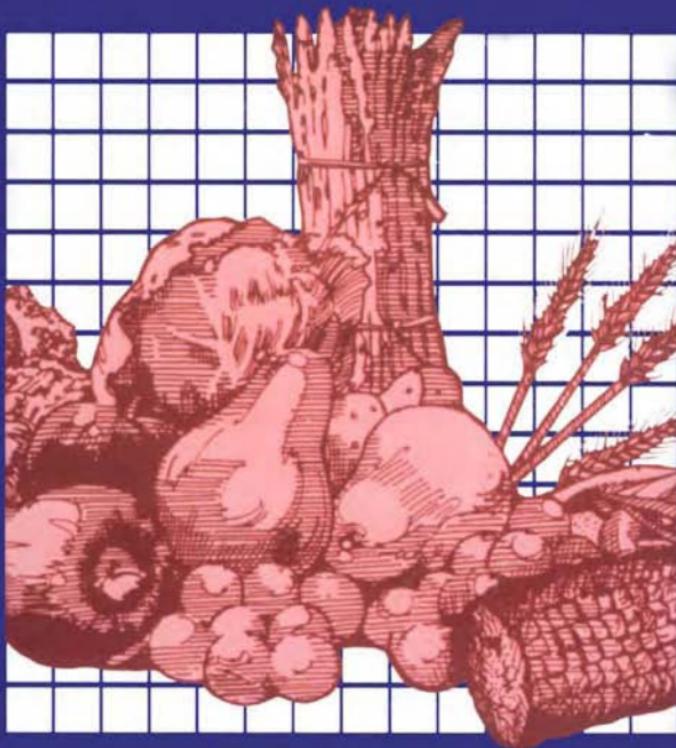


# PHYTOCHEMICALS AS BIOACTIVE AGENTS

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**Edited by**

Wayne R. Bidlack  
Stanley T. Omaye  
Mark S. Meskin  
Debra K. W. Topham



**CRC PRESS**

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# Preface

**E**PIDEMOLOGICAL evidence has consistently provided positive correlations between certain diets, specific foods, and disease expression. Diets rich in fruits, vegetables, and grains have been associated with disease prevention. During the assessment of the chemical components of these food groups, it became apparent that health benefits did not correlate solely with nutrient content. In fact, many phytochemicals were identified that displayed bioactivity in prevention of cancer, heart disease, and other diseases. Further evaluation of the mechanisms of action of these chemicals remains essential to our understanding of which chemicals are effective at low concentrations within the existing food supply.

Strategies used to identify bioactive phytochemicals are presented by in Chapter 1. The goal is to minimize wasted effort and improve on the systematic identification and characterization of the plant components. Ethnobotanical, chemical, ecological, and anatomical information can be used singly or in combination to identify which species and what tissues might provide valuable biologically active phytochemicals. Examples of various plants examined include *Sorghum* species (sorgoleone), *Artemisia annua* (artemisinin), and *Hypericum* species (hypericin). The use of modern analytical instrumentation and a broad array of bioassays are used to identify these agents, but more are needed.

Chapter 2 demonstrates the use of Quantitative Structure-Activity Relationship (QSAR) analysis and molecular modeling of bioactive phenolic compounds from plants to determine new directions for identification of other active agents. Data is presented correlating the inhibitory activities of tannins and lignans against HIV-1 Reverse Transcriptase and hDNAP- $\alpha$  with  $\mu$ , log MW, II<sub>b</sub>, and I. Structural similarities of estrogenic isoflavonoids with natural estradiol and the synthetic diethylstilbestrol, and antiestrogenic flavonoids with synthetic antiestrogenic tamoxifen and toremifene are compared. The

heat of formation and other physiochemical parameters are shown to predict the antioxidant activities of vitamin E analogs. Curcumin, which inhibits DNA synthesis, transcription, translation, and different enzymes involved in signal transduction and free radical formation, has been determined to be an effective chemopreventive agent against several chemical carcinogens. Through systematic investigation using the methodologies described, it is believed that new applications can be found for old remedies and new plants.

Chapter 3 presents information on plants in the family Cruciferae and genus *Allium*, which protect against carcinogenesis by altering carcinogen metabolism. The active ingredients include glucosinolates, isothiocyanates, indole-3-carbinol, and diallyl sulfides. Glucosinolates are hydrolyzed by the enzyme myrosinase to isothiocyanates and other products. The isothiocyanates are potent inhibitors of lung and esophageal tumorigenesis in a variety of models. These agents inhibit cytochrome P450, the phase 1 system involved in carcinogen activation, and induce phase 2 enzymes involved in carcinogen detoxification. Isothiocyanate induced apoptosis may also contribute to chemoprevention. Indole-3-carbinol appears to be an effective inhibitor of carcinogenesis when administered before or concurrently with the carcinogen, but may be a promoter when provided after carcinogen administration. *Allium* thiols, such as diallyl sulfide, effectively inhibit chemical carcinogenesis produced by nitrosamines, hydrazines, polycyclic aromatic hydrocarbons, and others. In addition, carotenoids and curcumin have anticarcinogenic activity in animal studies. Phytoene, and other carotenoids, may be more active than  $\beta$ -carotene. Thus, the protective effect of many phytochemicals may occur by modifying enzymes involved in carcinogen metabolism.

The randomized controlled human trial provides the final and most critical step in evaluating intervention agents that may favorably influence human health. Chapter 4 describes the fundamental design, conduct, and interpretation of clinical trials that should provide cost-effective outcomes for final consideration.

There is increasing interest in using phytochemicals to optimize gastrointestinal tract (GIT) health and function. The GIT provides habitat for more than 400 species of bacteria. Chapter 5 presents data noting that the inclusion of fermentable fibers, such as inulin and oligofructose, into the diet enhances the densities of beneficial bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp. Not only are detrimental bacteria decreased, but also mucosal growth is stimulated, digestive and immune functions of the small intestine are increased, and secretion of hormones that stimulate GIT growth is enhanced. These changes also decrease the reductive enzyme activities implicated in carcinogenesis. Thus, fructooligosaccharides, and other fermentable fibers, are suitable for managing the GIT during development, maturity, and senescence and are capable of enhancing recovery after disturbances such as diarrhea.

Chapter 6 provides clear insight into the numerous phytoantimicrobial (PAM) agents, such as phenolics from essential oils, terpenes from spices, saponins and flavonoids from fruits and vegetables, and phytoalexins from herbs. These agents have been used in food preservation, but may also provide health benefits. Thiosulfinates elicit a broad-spectrum activity against bacteria, fungi, viruses, and parasites. Some of these compounds are discussed in other chapters relative to other bioactive functions. Many PAM agents provide dual functions, such as natural PAM-colorants (e.g., tumeric), PAM-flavorants (e.g., cinnamic aldehyde), PAM-antioxidants (e.g., allyl isothiocyanates), and PAM-Nutraceuticals (e.g., flavonoids from cranberry). The exciting potential is for food technologists to design “specific tailor-made” PAM compounds incorporating nutraceutical advantage while enhancing food safety and preservation. The author emphasizes the multifunctional effects and potential applications for PAM agents in food processing.

Various phytochemicals have been suggested to prevent cancer. The antimutagenic and anticarcinogenic effects of tea and tea constituents, such as the catechins, have been consistently reported in animal models. The evaluation of human epidemiological studies examining tea consumption and cancer risk have provided equivocal results. Three clinical intervention trials are presented in Chapter 7 evaluating the protective effects of tea on cancer in high-risk populations. A mixed tea preparation indicated positive effects in treating oral leukoplakia, including a decrease in DNA damage and inhibition of cell proliferation. Tea also decreased carcinogenic metabolites and may thereby prevent lung cancer in cigarette smokers.

Isoflavones have multi-effects in health protection. Chapter 8 discusses the estrogenic and proliferative activities of genistein. Genistein was determined to bind to the estrogen receptor at a 100-fold lower affinity than estradiol. *In vitro*, genistein enhances the proliferation of estrogen dependent (MCF-7) human breast cancer cells as well as the estrogen-responsive gene, pS2. When implanted in ovariectomized athymic mice, genistein sustained the growth of the MCF-7 cell tumors in the absence of estrogen. Thus, genistein can act as an estrogen agonist.

Carotenoids and curcumin contain natural antioxidant activities. Several natural carotenoids have been shown to have anticarcinogenic activity in animal studies. Chapter 9 describes differences in carotenoids. Some of them have higher potency than  $\beta$ -carotene.  $\alpha$ -carotene demonstrated higher activity than  $\beta$ -carotene in suppression of tumorigenesis in skin, lung, liver, and colon. Other carotenoids also have higher anticarcinogenic activity than  $\beta$ -carotene. Lycopene and  $\beta$ -cryptoxanthin may activate the tumor suppressor RB gene. The authors present a brief discussion about insertion of the crtB gene into mammalian cells to encode for the phytoene synthetase. Phytoene was then detected, and the resulting cells displayed an increased resistance to oxidative stress. Curcumin, the yellow pigment of tumeric, significantly inhibited the

tumor promotion process of the two-stage mouse skin tumors. A combination of carotenoids and curcumin may enhance their efficacy to prevent cancer.

Alfalfa saponins are multicomponent, triterpene mixtures of glycosides of medicagenic acid, zanhic acid, hederagenin, and soyasapogenols. Chapter 10 characterizes many of the bioactive properties of these agents, including fungitoxic, hemolytic, membrane polarizing, and cholesterol binding. The alfalfa saponins vary in concentration by season, environmental stress, and plant part used.

Saw palmetto (*Serenoa repens* (Batr.) Small) is a native of plant of the Southeastern United States. Chapter 11 presents evidence that extracts may be useful in treating benign prostatic hyperplasia. Initial reports have suggested that beta-sitosterol may be the active ingredient, although numerous other phytochemicals, such as tocopherols, tocotrienols, and fatty acids, were also present. Genetic variation and geographical location affects the composition of the saw palmetto fruit. Continued evaluation will enhance our understanding of the mechanism of action.

The role of cholesterol reduction in the prevention and maintenance of atherosclerosis and coronary heart disease is well established. Health claims associated with garlic to beneficially lower cholesterol are numerous, but clinical trial data are inconsistent. Chapter 12 describes several sources of heterogeneity among the trials, such as normocholesterolemic to hypercholesterolemic; variations in garlic preparations such as fresh, dried, aged, extracts, and oils; short-term studies, etc. Eliminating seriously flawed data, the evidence may suggest only marginal effects of garlic on cholesterol levels if used as a single therapy. Further data are needed to establish optimal dosage, preparation form, frequency, and dose determinations.

Chapter 13 describes current information on the bioactive components of rice bran and rice oil. A few of the active ingredients include tocopherols, tocotrienols, phytosterols,  $\gamma$ -oryzanol, polyphenols, and other compounds. To maintain the bioactivity of these compounds, inactivation of hydrolytic and oxidative enzymes and decreased microbial loads must be used to stabilize the rice bran. The author suggests that the biological effects reported for rice bran and rice bran oil may result from the synergistic interaction of multiple bioactive agents.

The closing chapter identifies additional classes of phytochemicals not covered previously. The basic principles of food science and technology, processing, and biotechnology were noted to provide ample opportunity to create functional foods that may deliver these bioactive phytochemicals, equal to or better than the original source. Functional food products from around the world were identified. The development of new products in this area will be exciting and may contribute to a healthier life span.

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# **Strategies for the Discovery of Bioactive Phytochemicals**

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## **INTRODUCTION**

**P**HYSICOCHEMICALS with biological activity have had great utility as pharmaceuticals and pest-management agents. Through the 19th century and into the first half of the 20th century, the primary strategy for discovery of plant compounds with these uses was determining the active ingredients of plants with reported medicinal or pesticidal properties. Pharmaceuticals, including salicylic acid and morphine, and pesticides, such as the pyrethroids and rotenone, are examples of the fruits of this strategy (Duke, 1991; Lydon and Duke, 1989; Pachlatko, 1998; Robbers et al., 1996).

Although this historical approach is still used, it reached the point of diminishing returns decades ago. Considering the large number of plant-derived products, different human populations co-distributed across the globe with different flora have probably discovered very few of the potential uses of plant products in medicine and agriculture. In the latter half of this century, the medicinal and agricultural chemistry industries have become increasingly dependent on purely synthetic approaches to product discovery, thus reducing their interest in natural products, despite the virtually untapped biological and chemical potential of natural products. Molecular design around a molecular target site is a commonly used synthetic chemistry approach that has been somewhat successful in pharmaceuticals, but much less productive in pesticide discovery. Combinatorial chemistry, in combination with high throughput screening, has further expanded the potential of the solely synthetic approach. This strategy appears to be currently favored by many companies. However, within the past few years, a resurgence of interest

in botanical sources of new medicines, nutriceuticals, and other bioactive compounds has emerged.

This renewed interest is due to several factors, including the realization that nature has already selected for biological activity, that many botanical compounds have yet to be discovered, and that relatively few known compounds have been adequately characterized biologically. Furthermore, modern analytical instrumentation and improved microbioassays have made discovery of these compounds less time consuming and laborious. New strategies other than exploiting anecdotal ethnobotanical lore must be used to more fully explore the plant world for compounds that can be used directly or as molecular leads for pharmaceutical and agricultural products. In this chapter, we briefly describe the ethnobotanical approach and concentrate on alternative strategies of discovery.

## **STRATEGIES FOR CHOOSING A PLANT SPECIES OR PLANT TISSUE**

### **ETHNOBOTANICAL APPROACH**

Most of the medicines of previous centuries were of botanical origin, products of centuries of ethnobotanical lore. These botanical remedies were generally effective, although they contained many inert compounds in addition to the active compound(s). The advent of modern organic chemistry and the reductionist concept of a single active ingredient led to the discovery and exploitation of many single bioactive compounds from plants that are now used for medicinal or pest-management purposes. These discoveries are well documented in numerous reviews (e.g., Carlson et al., 1997; Lydon and Duke, 1989). Useful drugs from the ethnobotanical lore include aspirin, quinine, camphor, and digitalis. Examples of pesticides discovered by this approach are the pyrethroids, rotenone, and strychnine.

The ethnobotanical approach is still successfully used. For example, the antimalarial drug artemisinin (Figure 1) was relatively recently found by Klayman (1985) to be the active principle from the ancient Chinese malarial remedy *qinghaosu*, a formulation of *Artemisia annua* L., commonly known as annual wormwood in North America. Artemisinin is now being produced from plants in commercial quantities.

The ethnobotanical lore has not been sufficiently explored. Most large pharmaceutical companies still commit some of their research to this strategy (Shu, 1998), and there are some companies, albeit relatively small ones, that base their entire drug discovery program on ethnobotanical approaches (e.g., Carlson et al., 1997). A small portion of our research program involves following up ethnobotanical leads. Although the ethnobotanical approach has been

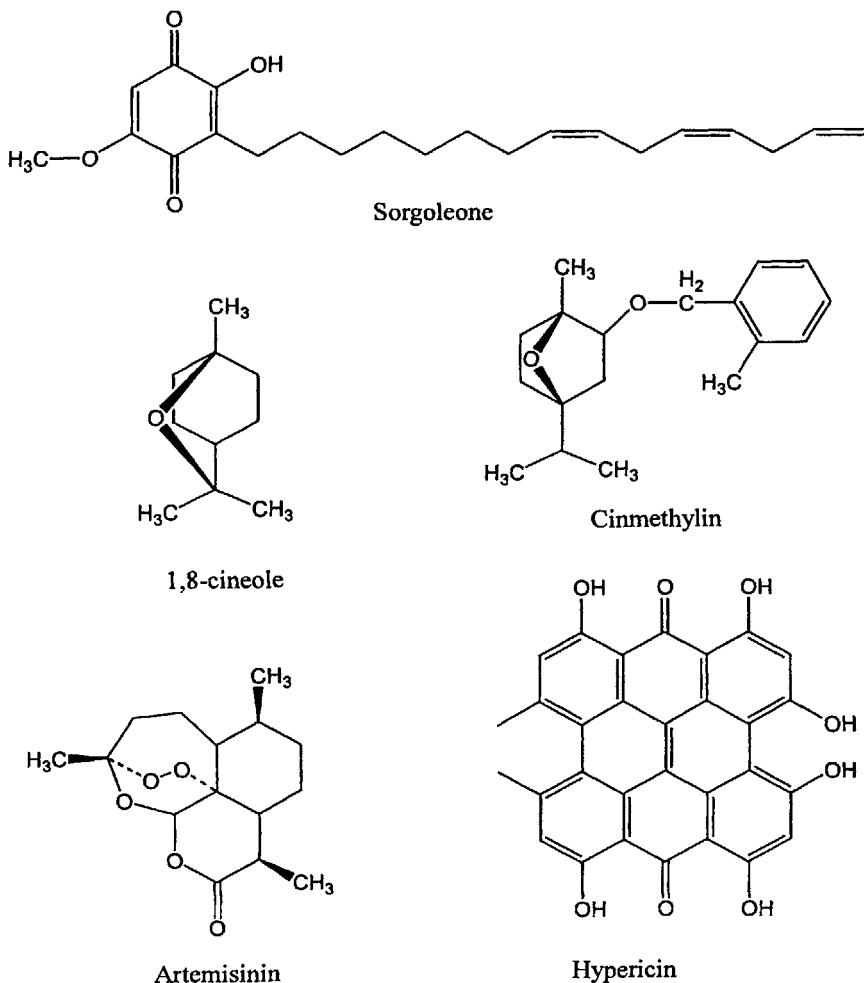


Figure 1 Structures of compounds mentioned in the text.

rewarding, it may have reached the point of diminishing returns. Other approaches for lead identification have been underexploited and, perhaps, may offer greater potential at this time. Our discovery strategies have focused more on these approaches.

## CHEMICAL ECOLOGY APPROACH

During the last half of the 20th century, chemical ecology has become a recognized subdiscipline: Understanding the chemical interactions between

different plant species, and between plants and other organisms, has resulted in the discovery of bioactive compounds with potential uses for humans. For example, a very potent natural herbicide was discovered through studies of the chemical ecology of *Sorghum* species.

Certain species of *Sorghum* are often chosen as a summer annual cover or green manure crop because of their rapid growth and ability to suppress weeds (Einhellig and Rasmussen, 1989; Forney et al., 1985; Putnam and DeFrank, 1983; Weston et al., 1998). The noxious weed johnsongrass [*Sorghum halepense* (L.) Pers.] also possesses the ability to chemically retard the growth of competing plant species (allelopathy) (Forney and Foy, 1985).

*Sorghum* roots exude large quantities of compounds with potent phytoinhibitory activity. Forney and Foy (1985) first noticed a yellow-colored root leachate that increased in toxicity with increasing plant age up to six weeks. Netzley and Butler (1986) first identified the major phytotoxic constituent in this root exudate produced by living sorghum seedlings as sorgoleone (Figure 1), a hydrophobic long chain benzoquinone. Sorgoleone is the major constituent in the root exudate, present in nearly pure form, with more than 85% consisting of sorgoleone and the remainder consisting of minor related components. Later, sorgoleone was determined to inhibit photosynthesis at concentrations less than 50  $\mu\text{M}$  (Einhellig et al., 1993).

Root exudates of various sorghum species and accessions contain predominantly sorgoleone and also a structurally related compound that is biologically active, ethoxysorgoleone (Rimando et al., 1998), along with numerous other compounds that vary in bis-allylic bonding in the side chain or ring substituents. In bioassays using isolated photosynthetic membranes of chloroplasts, sorgoleone was a more potent inhibitor of electron transport in photosystem II (PSII) as measured by oxygen evolution and chlorophyll  $\alpha$  variable fluorescence than were synthetic PSII inhibitor herbicides evaluated (Gonzalez et al., 1997; Nimbal et al., 1996). Sorgoleone is a competitive inhibitor of other photosynthetic inhibitors, such as diuron and metribuzin, and binds at a similar  $Q_B$ -binding site within the D1 protein of the secondary electron acceptor. Using 3-D computer imaging analysis and evaluating the PSII binding site, sorgoleone was recently found to fit within the  $Q_B$  site in a manner almost identical to that of plastoquinone, its natural electron acceptor, providing a logical explanation for its strong inhibition of electron transport at that site (Czarnota et al., 1998). Efforts are underway to use molecular genetics to impart and/or enhance the production of sorgoleone in crops, with the ultimate objective of reducing the use of synthetic herbicides.

Certain rice varieties apparently produce compounds that suppress competing weeds (Olofsdotter, 1998). We are currently using bioassay-directed isolation methods (see below) to discover the active herbicidal compounds in these rice varieties in order to more effectively manipulate production of these compounds by genetics or other means.

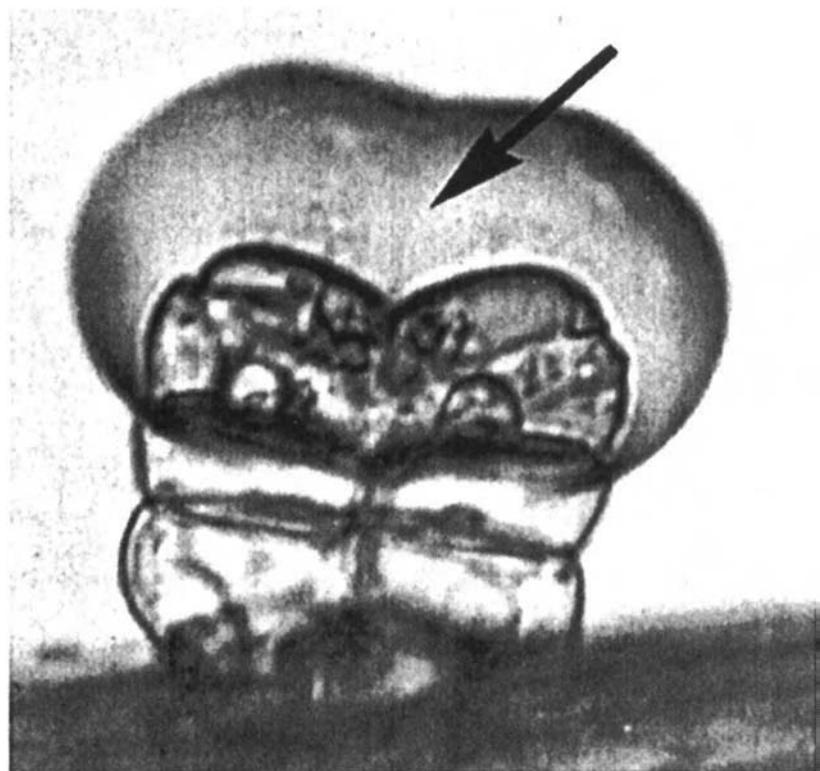
There are many other examples of chemical ecology studies leading to the discovery of potentially useful natural products from plants (c.f., Hedin et al., 1997).

## ANATOMICAL CLUES

Plants often compartmentalize, sequester, or secrete bioactive compounds from specialized tissues and/or cells. Structures commonly associated with secondary compound accumulation in plants are glandular trichomes, laticifers, idioblasts, resin canals, and nectaries. The primary driving forces for the evolution of these different structures are the needs for efficient synthesis and delivery of secondary products to enhance interaction with other organisms and for avoidance of autotoxicity. In other cases, highly active compounds can be found in specialized cell layers or epidermal cell secretions. Information on the anatomical specialization at the subcellular, cellular, tissue, or organ level related to the synthesis and storage of these compounds can provide clues as to function and activity. Several examples of anatomical specialization related to the production of compounds with high levels of biological activity are provided below.

Annual wormwood (*Artemisia annua* L.) is covered with peltate glands, composed of a club-like group of stalk cells covered with an elastic cuticle (Duke and Paul, 1993; Ferreira and Janick, 1995). The elastic cuticle engorges with terpenoids produced by the stalk cells, resulting in a balloon-like covering over the stalk (Figure 2). Later, the cuticle splits to spill its contents across the epidermis. Compounds distributed within the plant and on the plant surfaces in this way probably act as antimicrobial and antiherbivore (including insects) agents. Avoidance of autotoxicity can be another reason for such a production and distribution system.

One of the terpene components of annual wormwood is the antimalarial drug, artemisinin. We found this compound and related natural and synthetic analogues to be highly phytotoxic (Dayan et al., 1999; Duke et al., 1987; 1988). Annual wormwood itself was equally sensitive to artemisinin (Duke et al., 1987). This led us to reason that the plant needs a highly specialized structure, such as a peltate gland, to sequester this toxicant for autotoxicity avoidance (Duke, 1994; Duke et al., 1994). Thus, we expected the most potent phytotoxin, artemisinin, to be found only in the glands. This hypothesis was confirmed by a glandless mutant that contained no artemisinin or artemisitene (Duke et al., 1994). The mutant also contained few or none of the monoterpenes found in the glanded biotype (Tellez et al., 1999), suggesting that they, too, might be autotoxic to annual wormword. In fact, many of these compounds are reported phytotoxins (Duke et al., 1988; Duke, 1991; Lydon and Duke, 1989). We also found that when the glands of fresh leaves of annual wormwood are extracted with a five-second immersion in chloroform, so as to only



**Figure 2** Peltate gland of *Artemisia annua*. Arrow denotes the subticular space filled with terpenoids. (Ferreira and Janick, *Floral Morphology of Artemisia annua Special Reference to Trichomes*. *IJPS* 156(6):807–815, Figure 5A. Copyright © 1995 The University of Chicago Press.)

extract the glands, but not the leaf tissue, virtually all of the artemisinin and artemisitene were extracted (Duke et al., 1994). Clearly, these and other compounds are produced exclusively by glandular cells. This finding has implications for those interested in more efficient extraction methods for such high-value products as artemisinin, in producing higher yielding chemotypes of such species, and in generating such compounds in cell or tissue cultures of fairly undifferentiated cells. In the case of artemisinin, there has been little or no success in production of this high-value compound in cell or tissue culture (Ferreira and Duke, 1997).

Several species of *Hypericum* (St. John's wort) produce hypericin (Figure 1), a red, polycyclic naphthodianthrone, photodynamic pigment with several pharmaceutical properties, including antiviral and anticancer activity (e.g., Lavie et al., 1995; Koren et al., 1996), a treatment for prevention of macular degeneration (Kimura et al., 1997), and in crude preparations of *H. perforatum*

L., standardized by hypericin content, as a treatment for depression (Upton et al., 1997).

The photodynamic properties of hypericin make it generally cytotoxic. In fact, consumption of large quantities of weedy *H. perforatum* L. is a serious problem to livestock, due to the photodynamic nature of hypericin (Giese, 1980), although, at recommended doses for treatment of depression, there is little evidence of photosensitivity in humans (Brockmöller et al., 1997). Hypericin is also an effective photoactive insecticide (Knox et al., 1987) and phytotoxin (Knox and Dodge, 1985). When fresh leaves of *H. perforatum* L. are floated on a solution of hypericin under bright light, they are damaged (unpublished data). Thus, the plant must have some method of protecting itself.

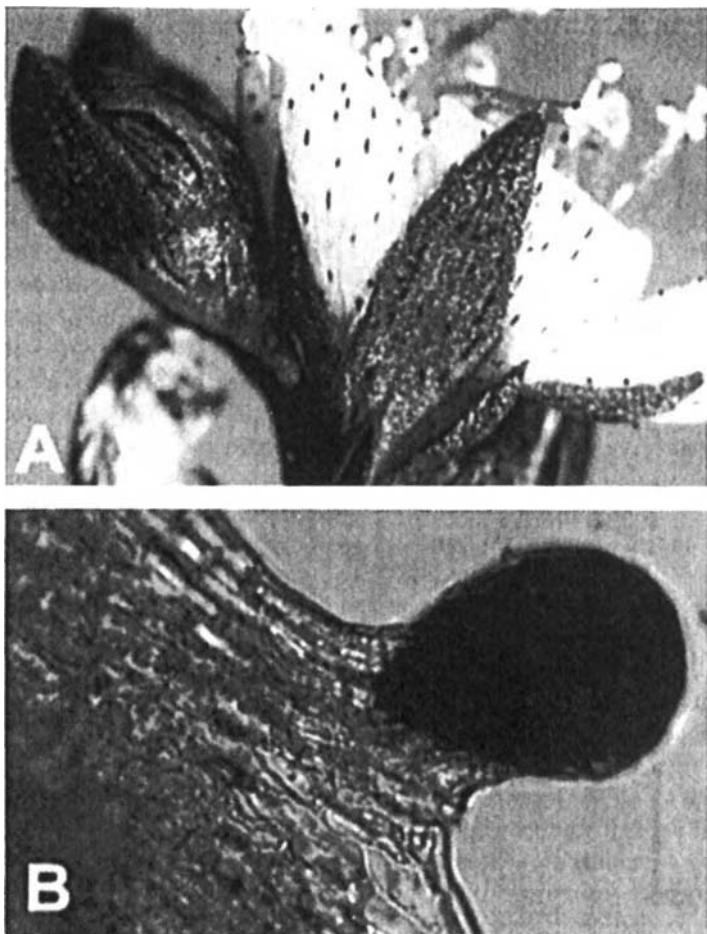
Considering the greatly increased interest in this compound, little is known of the anatomy or physiology of the plant structures that produce hypericin. Being a red dye, visual observation indicates that hypericin is localized in glandular structures dotting the leaves, flowers, sepals, stamens, and stems [Figure 3]. There appear to be two general types of glandular structures that produce the compound. *H. perforatum* L. has more flattened, "nodular" structures (Curtis and Lersten, 1990), whereas other species such as *H. hirsutum* L. (Knox and Dodge, 1985) and *H. punctatum* Lam. have stalked, pigmented nodules [Figure 3].

The "nodules" do not have the structure of most secretory glands, in that there does not appear to be any subcuticular accumulation of any product (Knox and Dodge, 1985; Curtis and Lersten, 1990). Instead, the hypericin-containing structures appear to be composed of a solid mass of cells (Curtis and Lersten, 1990). The outer cells of the mass are flattened to form a sheath around a core of more isodiametric cells. Our preliminary findings are that hypericin is localized entirely within the vacuole of these central cells.

Although the exact function of hypericin for the producing plant is unknown, the location and biological activity support the view that it is a plant defense compound. Indeed, some insect larvae that feed on *H. perforatum* do so only at night and hide in the dark during the day, while others avoid feeding on the glands (Fields et al., 1991). Its antimicrobial activity could protect the plant from plant pathogens (Giese, 1980), although its apparent distribution within the plant does not provide strong support for this theory.

Roots possess a plant surface that comes in contact with potentially detrimental organisms. Many highly potent compounds can be exuded by roots. For example, in the chemical ecology of *Sorghum* species mentioned above, a clue that these species might be making a compound with strong biological activities might have been the presence of droplets containing as much as 90% sorgoleone and related compounds exuded or secreted from root hairs.

In the cases mentioned above, the anatomical distribution was discovered after the compound was isolated and then chemically and biologically characterized. The reverse sequence of anatomical examination, followed by isolation



**Figure 3** (a) Leaves and sepals of *Hypericum punctatum* with hypericin-containing trichomes dotting their surface. (b) Light micrograph of hypericin-containing trichome of *H. punctatum*.

and characterization of compounds from structures of interest, should be a valid discovery strategy for new compounds. Clearly, the compounds sequestered, compartmentalized, or secreted by plants should be studied for their biological activities. Species that partition relatively large portions of their biomass into such structures and processes might be expected to be good candidates for the discovery process.

#### TOOLS FOR DETERMINATION OF ACTIVE COMPOUNDS FROM A PLANT

Once a plant species is chosen, the process of dereplication (isolation of

and identification of active components) begins. This process involves the integration of bioassays, analytical instrumentation, and informatics. After active compounds are discovered, their activity can often be optimized by generation of synthetic analogues and use of computational chemistry-based quantitative structure-activity relationship (QSAR) analysis.

## BIOASSAYS

As mentioned earlier, when one surveys the phytochemical literature, the emphasis has clearly been on chemistry, rather than on biology. Thus, few chemically characterized natural compounds have been extensively tested for an array of biological activities. When known compounds are tested in new bioassays, new potential uses of the compounds can be evaluated (e.g., Duke et al., 1987; Schrader et al., 1998). Bioassays can also be used to direct the isolation of new bioactive compounds from plants (Choudhary and Atta-ur-Rahman, 1997). We will concentrate on the latter approach, but first we would like to briefly discuss bioassays in general.

Bioassays can range from molecular assays to whole-organism assays. Each has its advantages, depending on the strategy and one's objectives. In general, target site-specific assays lend themselves to synthetic chemistry approaches for pharmaceutical and pesticide discovery, particularly when myriad compounds are generated by combinatorial chemistry techniques. Such assays are often easier to automate and miniaturize for high-throughput screening.

Natural product discovery efforts usually produce relatively few compounds, so there is less need for high-throughput screening. Nevertheless, an assay at the molecular level is often useful with phytochemicals when bioassay results, molecular structure, or ethnobotanical or chemical ecology information indicates a mechanism or molecular site of action. Miniaturized whole organism bioassays are optimal for most natural product-based discovery processes for pesticides or antimicrobial pharmaceuticals. Considering that the test organism can have many potential molecular target sites, this strategy minimizes the risk of missing an active compound. Furthermore, it maximizes the possibility that a previously unknown molecular site of action will be discovered, a highly desirable outcome from a patent protection standpoint and as a new tool in combating the evolution of resistance.

The amount of compound available for bioassays is often very limited in natural product programs, further intensifying the need for microbioassays. This limitation is not a problem with microorganisms. For example, we developed a semiautomated microtiter plate based bioassay in a discovery program to identify algicides that will selectively limit cyanobacteria (blue-green algae) responsible for undesirable flavors in fish produced in aquaculture (Schrader et al., 1997). Results from this inexpensive bioassay can be obtained within a few days. Currently, we use two microbioassays for antifungal agent discovery: a rapid bioautography assay (Wedge and Kuhajek, in press) and a 96-

well microbioassay (Wedge and Kuhajek, 1998). Bioassays for herbicides can also be conducted in microtiter plates (e.g., Dayan et al., 1999), the number of wells of the plate depending on either or both the amount of test chemical available and the size of the seed of the tested species.

Perhaps the best approach for finding new compounds is bioassay-directed isolation. With this method, each fraction is bioassayed, and those with some threshold of activity are further fractionated and bioassayed, etc., until a pure, active compound is isolated (Figure 4). A microbioassay with a whole organism is highly desirable with bioassay-directed isolation, so that all potential molecular sites of action can be tested simultaneously. Although bioassay-directed isolation is the best strategy for finding new bioactive molecules, this method is labor intensive and costly. Without a method to differentiate between known compounds and new compounds, this procedure can be very frustrating. As discussed above, bioassay databases can be used to reduce the cost of pursuing known compounds. However, modern analytical instrumentation may be most effective in streamlining bioassay-directed isolation.

## ANALYTICAL INSTRUMENTATION

Bioassay-directed discovery of natural products entails isolation and purifi-

### Bioassay-directed isolation

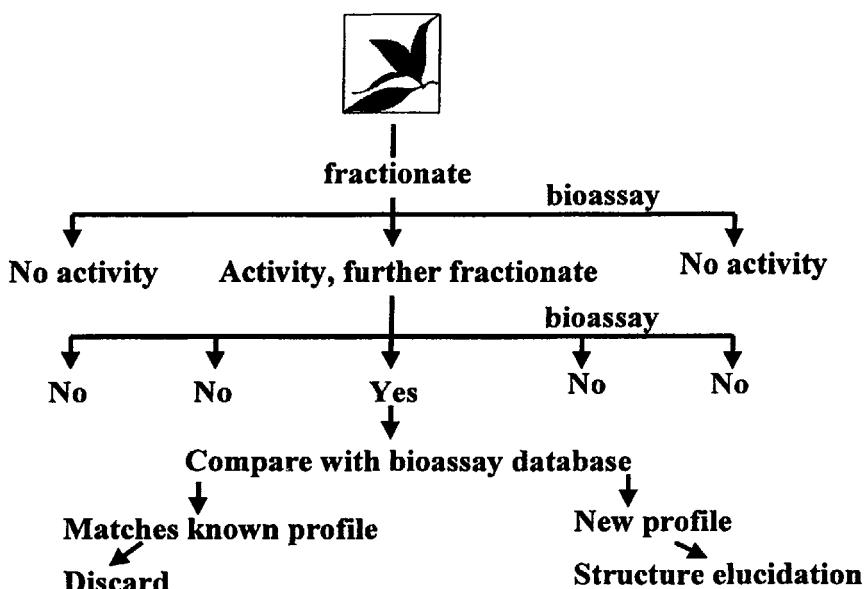


Figure 4 Bioassay-driven discovery strategy for new phytochemicals.

cation of the secondary metabolites using various separation techniques followed by structural identification through spectroscopic means. In the past decade, high-performance liquid chromatography (HPLC) has been the standard laboratory tool in separating mixtures of compounds in an extract. However, the only information that relates to the identity of compounds in a mixture provided by HPLC is retention time (RT) and specific responses of the compounds from detectors (refractive index, ultraviolet light, fluorescence, electrochemical, radiochemical, and photodiode array spectrophotometric) used on-line with HPLC. Identity of known compounds can sometimes be concluded from this type of information, but no detailed structural information is obtained from this method. Furthermore, the RT is of no use as a compound marker in cases where compounds in a mixture co-elute.

Technological advances in analytical instrumentation made possible the coupling of separation and spectroscopic methods. The hyphenated techniques of liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR) spectroscopy have clear advantages over conventional isolation-structure elucidation procedures, which are labor intensive, time-consuming, and require larger quantities of sample for analysis.

LC-MS has gained attention as a convenient method for identification and structure determination, as well as quantitative analysis of compounds in complex matrices with the development of interfaces between HPLC and MS, particularly electrospray ionization (ESI), thermospray (TSP), and atmospheric pressure chemical ionization (APCI) (e.g., Iwabuchi et al., 1994; Siuzdak, 1994; Zhou and Hamburger 1996). LC-MS makes possible the analysis of non-volatile compounds that would not be amenable to analysis using gas chromatography-MS. LC-MS provides the molecular weight of compounds, and further structural details can be obtained with the more powerful LC-MS-MS systems which provide information on the characteristic fragmentation pattern typical of a compound. In LC/MS screening of the bioactive methanol extract of *Rollinia mucosa*, 40 known and four new acetogenins (determined to have molecular weights 578 and 604 Da and possessing a C-4 hydroxyl group) were identified without having to isolate the compounds (Gu et al., 1997). Using LC-MS in conjunction with LC-NMR, direct identification of antibacterial sesquiterpene lactones from a partially purified extract of *Vernonia fastigiata* was achieved without isolation of individual compounds (Vogler et al., 1998).

The merits of LC-MS are evident. However, this technique does not always provide unambiguous structural identification, particularly with compound isomers. In this case, LC-NMR is the preferred method, providing proton multiplicities and coupling information. NMR on-line with HPLC was introduced in the late 1970s, and, with the advent of higher field strength spectrometers, improved solvent suppression techniques, and decreasing cost of deuter-

ated solvents, LC-MS has become more widely used (Albert, 1995; Lindon et al., 1995). Further development allowed acquiring 2D-NMR experiments in a stopped-flow mode, thereby enabling full characterization of target compound(s) in a mixture. Although LC-NMR is being employed primarily in studying the metabolic fate of drugs (Lindon et al., 1997), it has also found application in the analysis of natural products. HPLC-NMR was used in the structural identification of the photo-isomerization product of azadirachtin, an insect-antifeedant and growth-regulating substance from the seeds of the neem tree (Johnson et al., 1994). An extract from 250 mg of dried leaves of *Zaluzania grayana* was analyzed by LC-NMR to elucidate the sesquiterpene lactones found in the glandular trichomes (Spring et al., 1995). In the analysis of naturally occurring vitamin A and synthetic vitamin A acetate isomers, LC-NMR was found to be a valuable method in characterizing two overlapping peaks of isomers based on the difference in their <sup>1</sup>H-NMR spectra (Albert et al., 1995). Furthermore, in the same study, it was determined that a 40% saving of analysis time was achieved (two hours of on-line LC-NMR compared to 3.5 hours of HPLC separation and off-line NMR measurements). Many compounds in complex matrices are not separated using normal or reverse-phase HPLC methods. Thus, further development has taken place and has found application in coupling NMR with supercritical fluid extraction (Albert et al., 1994), capillary electrophoresis (Wu et al., 1994), and centrifugal partition chromatography (Spraul et al., 1997).

Sufficient structural information can usually be obtained from <sup>1</sup>H-NMR spectra alone; however, in situations where a compound does not have hydrogen containing functional groups (e.g., sulfates, *N*-oxides) or where protons exchange with the solvent, unequivocal structural assignment may not be achieved. In this case, LC-MS can be used in conjunction with LC-NMR to determine compound structure. Clearly, the use of the doubly hyphenated system LC-MS-NMR is the most efficient method for complete structural elucidation of compounds in a mixture. HPLC-MS-NMR analysis of complex mixtures has been described for the analysis of xenobiotics in urine (Shockcor et al., 1996; Scarfe et al., 1997; Clayton, 1998) and a mixture of peptides (Holt et al., 1997). Undoubtedly, this technique will be extremely valuable in the study of natural products.

At the moment, the major problem encountered with LC-MS-NMR is the lack of integrated systems that would allow the chromatograph, NMR, and MS units to be controlled from a single console. However, this problem is being addressed, and work is being done currently to develop suitable software. The cost of the equipment is high, but this is offset by much higher efficiency achieved and by the detection of known compounds at the early stages of natural product discovery efforts.

## INFORMATICS

Considering the large number of known natural products from plants and

the even greater number that probably remain to be discovered, as well as the many potential biological activities that these compounds might have, acquisition and storage of chemical and related biological data are crucial components of a discovery program. Various commercially available databases for natural products and their chemical and biological activities exist (e.g., Buckingham and Thompson, 1997; Corley and Durley, 1994). Unfortunately, there is no universal data repository on this topic, so each discovery group must decide which commercially and publicly available information that it will use, as well as creating its own set of bioassay profiles from its own research efforts.

Previously unstudied organisms often produce already discovered compounds with known biological activity. In fact, some bioactive natural products, such as taxol (Strobel et al., 1996), have been found to be produced by both higher plants and fungal endophytes. Pharmaceutical and agrochemical companies that have had natural product-based discovery programs have found rediscovery of known compounds during replication to be a costly and time-consuming problem. This problem occurs even when a complete database is kept of the profiles of known compounds in the company bioassays. For example, an industrial discovery group using a discovery method like that in Figure 4 found that even with an extensive database to terminate further examination of compounds that matched known compounds in their bioassays, 72% of compounds that reached the structure determination stage were known compounds (Ayers et al., 1989). This result was with microbial natural products, but the problem is the same, regardless of the source.

This older informatics strategy may have eliminated new compounds that happened to have the same bioassay profiles as known compounds. It was an ongoing battle for any researcher to determine where thresholds should be set. If the bar was set too low, the "wonder drug" of the century might be lost, and, if too high, one could end one's days chasing known compounds. One of the more intensive examples of the bioassay database strategies for rediscovery avoidance was developed by researchers at the National Cancer Institute (Decosterd et al., 1994). Isolates were tested against 60 human cell lines representing seven major categories of human cancer to generate biological activity "fingerprints." Isolates with unique "fingerprints," as predefined by the researchers, are then pursued. This system seems to work well for the NCI researchers. However, most independent researchers have neither the time nor resources to develop, standardize, run, and analyze data for 60 different assays. Also, this method relies on biological information for the initial selection process. Such an approach means that many data are generated and analyzed before "known" suspects are eliminated. Lastly, one always has to be concerned with losing "trace" analogs that may be buried within complex isolates.

Modern instrumentation (see above) has reduced the need for an extensive database of bioassay profiles for known compounds. Using such methods,

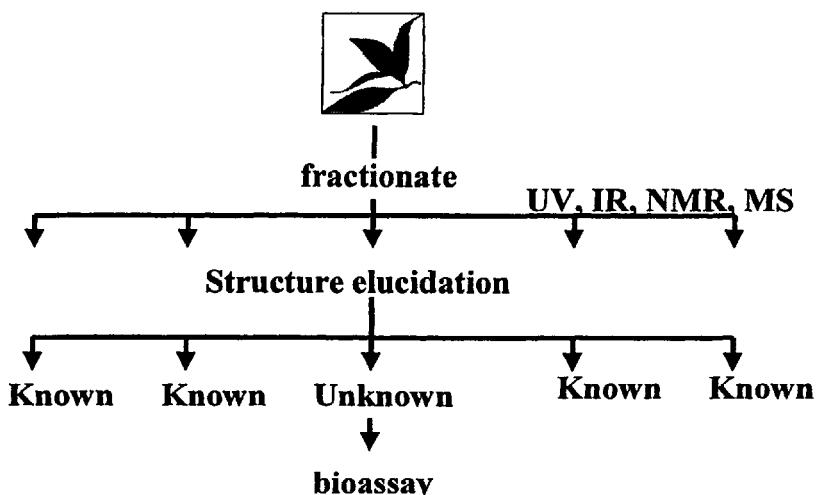
mixtures can be fractionated by liquid chromatography, with the fractions split for simultaneous bioassay and identification (e.g., Likhositawuid et al., 1993; Cui et al., 1998). Using such a method, fractions with known compounds with known activity in the bioassay(s) could be eliminated before bioassay; i.e., fractionation-driven bioassays (Figure 5). Automated chemical dereplication using tandem instrumentation, robotics, and extensive chemical database information (e.g., Hook et al., 1997; Whitney et al., 1998) will probably and frequently lead to the use of the bioassay only after the chemical structure of a compound is determined.

### STRUCTURE OPTIMIZATION BY COMBINATORIAL AND COMPUTATIONAL CHEMISTRY

Many bioactive natural products are derived from a plant's secondary metabolism. While their biological function(s) are often unknown, their existence must somehow be justified because plants expend energy synthesizing these molecules. Structurally, natural products have developed a complexity in carbon skeleton over time in order to address specific circumstances faced by the producing organisms at a particular time. This structural diversity has been and still remains an invaluable source of lead compounds in developing novel pharmaceutical drugs and agrochemical products.

Unfortunately, natural products are generally poorly suited for commercial

### Fractionation-driven bioassays



**Figure 5** Instrumentation-driven strategy for new phytochemicals.

use due to sub-optimal physical and biological properties, such as stability, volatility, lipophilicity, and selectivity. Furthermore, the natural structure may not be optimal for greatest activity, either because the molecular target site for a commercial product may be different than that for which it evolved in nature, or evolution of the molecular structure may have met an evolutionary impasse. Thus, optimization of structure for greater activity may be desirable. In fact, very few natural products are commercialized in their original forms. Most molecules require some level of structural optimization to increase their suitability as commercial products. For example, the commercial herbicide cinnmethylin (Figure 1) is much less volatile than the natural phytotoxin cineole (Figure 1) from which it was derived (Grayson et al., 1987). The lability of the cyclopropane moiety found in natural insecticidal pyrethrroids limited their use to an indoor environment. Structure optimization resolved this and other limitations associated with natural pyrethrroids and led to the development of virtually all pyrethrroids commercially available today (e.g., Fujita, 1995).

The first step in structure optimization is to produce an array of close analogs of the lead compound. Some of these might be natural compounds, but most are generally synthetic. The analogs can be generated either by conventional synthetic chemistry or by combinatorial chemistry. It is important that the compounds have a wide spectrum of activity in the bioassay used for optimization. Computational chemistry methods, such as traditional two- and three-dimensional quantitative structure-activity relationships (QSAR), and more powerful methods such as Comparative Molecular Field Analysis (CoMFA), are used to describe the molecular descriptors for the analogs. This information is then correlated with biological activity to determine the structural components responsible for activity. This information is then used to predict new structures with enhanced activity and optimal physical properties.

Some pharmaceutical research has recently focused on the peptidomimetic approach to developing novel drugs. This computation-intensive technique relies on high-resolution analysis of interesting target receptor/ligand complexes to design protein-like secondary structure mimetics. These molecules have a conformation similar to the receptor-ligand complex and act as competitive inhibitors. This concept is now being applied to derive peptidomimetic structures from bioactive natural products (Müller and Giera, 1998).

## CONCLUSIONS

We have briefly described the strategies for identification of plant species and/or plant parts that are most likely to have bioactive compounds of interest for use as pharmaceuticals or agrochemicals. Ethnobotanical, chemical ecological, and anatomical information can be used singly or in combination to provide clues as to what plant species and what tissues of those species might

be worth the major investment of time and resources to conduct a careful dereplication. Furthermore, information from these sources can provide valuable hints as to what types of biological activity the active compounds might have. Ethnobotanical leads are more likely to suggest pharmaceutical uses, whereas chemical ecology and anatomical information often lead to potential agrochemical uses. After a plant species is selected, the dereplication process begins. Rediscovery of known compounds has been the most costly aspect of this process. With modern informatics, miniaturized and automated bioassays, and tandem separation-NMR or MS/MS analytical instrumentation, dereplication can be much faster and more efficient than before, potentially eliminating known compounds before the bioassay step. After discovery of new lead compounds from plants, biological activity can be optimized by computational chemistry-based QSAR studies of analogues, both synthetic and natural.

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# QSAR and Molecular Modeling of Bioactive Phyto-phenolics

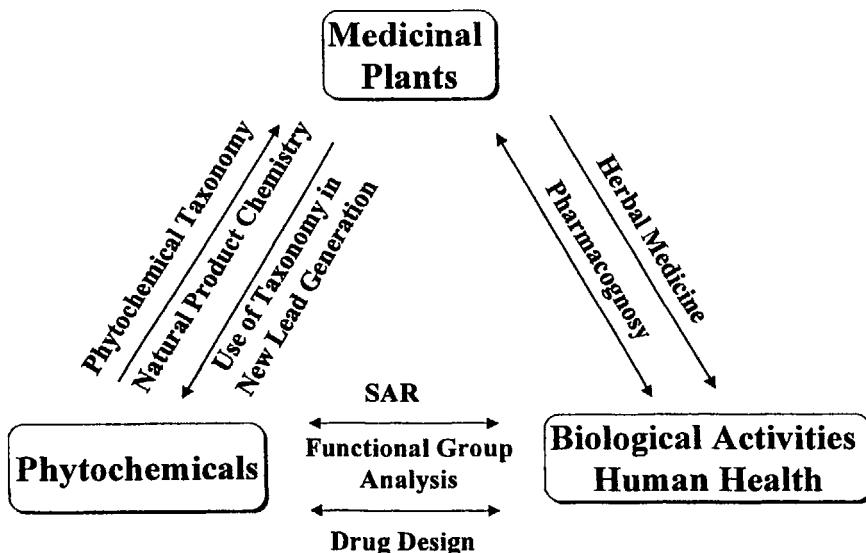
ERIC J. LIEN, SHIJUN REN

## INTRODUCTION

**O**n March 13, 1998, the *Los Angeles Times* had a front page news story showing that the incidence of all cancers declined an average of 0.7% a year since 1993 and that cancer death rates declined an average of 0.5% a year from 1990 to 1995. According to the report, health officials attributed these declines to early diagnosis, smoking cessation, and better medical treatment. While this may be part of the story about progress in modern medicine, it is important to point out that chemoprevention and adjuvant nutrition in cancer treatment may have contributed to the resultant reduced cancer incidence and increased cure rates in the general population and defined high-risk groups (Quillin and Williams, 1993; Quillin and Quillin, 1994; Diamond et al., 1997). Many compounds isolated from plants have been shown to have chemopreventive effects (Gao and Lien, 1991; Ren and Lien, 1997). More and more people all over the world are realizing the necessity of proper nutrition, exercise and rest, dietary supplementation of vitamins and chemopreventive agents, and the avoidance of carcinogenic agents, in reducing the risk of cancer.

Figure 1 indicates the triangular relationship among medicinal plants, phytochemicals, and human health. In herbal medicine (pharmacognosy), usually, crude herbal products or standardized extracts of medicinal plants are used in treating diseases.

In the past, phytochemicals like flavonoids have been used in identifying plants in taxonomy. One can go the other direction in using taxonomy in the



**Figure 1** The contributions of medicinal plants to human health.

generation of biologically active compounds of the same or related molecular structures.

In recent years, our group has been using the tools of quantitative structure-activity relationship (QSAR) and molecular modeling in drug design based on bioactive phytochemicals or synthetic compounds. Some of the examples will be shown in this chapter.

In this chapter, focus was placed on a special group of phytochemicals, namely phenolic compounds found in plants. Many phenolic compounds like lignans, tannins, isoflavonoids, flavonoids, vitamin E, and curcumin have been shown to have important biological activities. They are biosynthesized either from aromatic amino acids and/or other intermediates like malonyl-CoA (Balandrin et al., 1985). Lignans and tannins have been shown to have inhibitory activities against HIV-1 reverse transcriptase (HIV-1 RT) and human DNA polymerase- $\alpha$  (hDNAP- $\alpha$ ) (Chen et al., 1997). Phytochemicals like flavonoids, isoflavonoids, polyphenols, and many other antioxidants are receiving extensive investigation in terms of their roles in health and disease prevention (Cadenas and Packer, 1996; Rice-Evans and Packer, 1998). Genistein, one of the soybean isoflavonoids, has been shown to reduce stress-response-related gene expression, which may contribute to its anticancer activity (Zhou and Lee, 1998). Most recently, we utilized the calculated parameters including the heat of formation ( $H_f$ ), the energy of the highest occupied molecular orbital ( $E_{homo}$ ), and the energy of the lowest unoccupied molecular orbital ( $E_{lumo}$ ) to

correlate with the redox potentials of substituted phenolic compounds and antioxidant activities of vitamin E analogs (Lien et al., 1999).

The mechanism of growth inhibition of phenols with electron-releasing and electron-withdrawing substituents in mouse leukemia cells has been proven to be bifurcate (Selassie et al., 1998). In the case of electron-releasing substituted phenols, the toxicity is mainly dependent on radical formation (i.e., radical-mediated process), while, for electron-withdrawing substituted phenols, the toxicity is mainly mediated by hydrophobicity (i.e., hydrophobicity-mediated process). Curcumin and related compounds may inhibit tumor promotion by blocking the signal transduction resulting in apoptosis (Lin and Lee, 1995). In our laboratory, we have been using the tool of QSAR analysis and molecular modeling to elucidate the molecular bases of these phytochemicals toward their biological activities. The results obtained may provide new directions for molecular modification as well as for future new lead generation.

## LIGNANS AND TANNINS AS ANTIVIRAL AND ANTITUMOR AGENTS

The QSAR model proposed by Lien (Lien, 1987) has been successfully applied to phytochemicals isolated from various medicinal plants. The inhibitory activities against HIV-1 RT and hDNAP- $\alpha$  of 15 lignans and tannins have been correlated with physicochemical parameters and one indicator variable ( $\mu$ , log MW, H<sub>b</sub>, and I). It has been shown that there are different structural requirements for the inhibition of HIV-1 RT and hDNAP- $\alpha$ . From the overall shapes of 3-D structures, a T-shaped perpendicular ring system gives the best differential inhibition against HIV-1 RT, while a more complicated  $\pi$ -shaped ring system is associated with high inhibition against both HIV-1 RT and hDNAP- $\alpha$  (Figure 2) (Chen et al., 1997).

vs. HIV-1 RT (A)

$$\log 1/IC_{50} = 1.47I + 0.41\mu + 2.69 \log MW - 4.36 \quad (1)$$

$$n = 14$$

$$r^2 = 0.88$$

$$r = 0.94$$

$$s = 0.42$$

$$F_{3,10} = 27.38$$

$$p < 0.01$$

vs. hDNAP- $\alpha$  (B)

$$\log 1/IC_{50} = 0.13H_b + 0.92I - 7.00 \log MW + 19.58 \quad (2)$$

$$n = 15$$

$$r^2 = 0.85$$

$$r = 0.92$$

$$s = 0.53$$

$$F_{3,11} = 21.21$$

$$p < 0.01$$

B/A ratio (differential toxicity toward HIV-1 RT)

$$\log (B/A) = 0.14H_b + 10.36 \log MW + 0.17\mu - 24.07 \quad (3)$$

$$n = 14$$

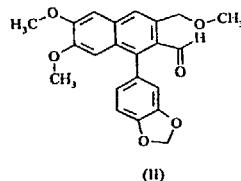
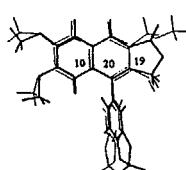
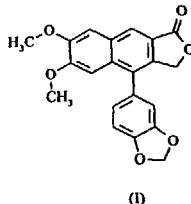
$$r^2 = 0.81$$

$$r = 0.90$$

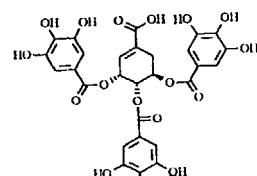
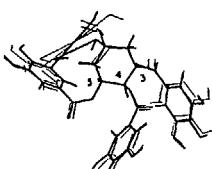
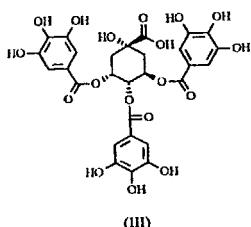
$$s = 0.49$$

$$F_{3,10} = 13.80$$

$$p < 0.01$$



(I) Retrojusticidin B (bold) and (II) phyllamyricin B (light) with selectivity against HIV-1 RT.



(III) 3,4,5-tri-*O*-galloylquinic acid (bold) and (IV) 3,4,5-tri-*O*-galloylshikimic acid (light) with high activities toward both HIV-1 RT and hDNAP- $\alpha$ .

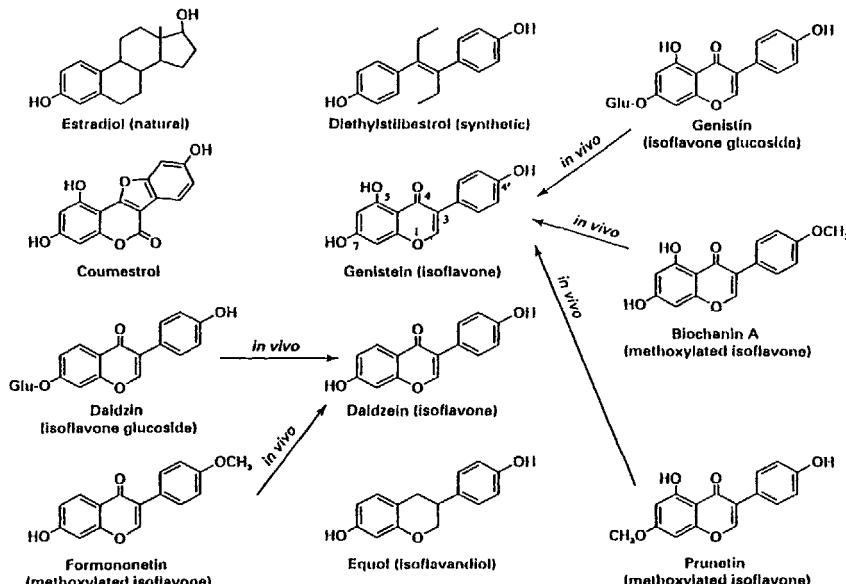
**Figure 2** Superimposed 3-D models of retrojusticidin B (bold) (I) and phyllamyricin B (light) (II), and 3,4,5-tri-*O*-galloylquinic acid (bold) (III) and 3,4,5-tri-*O*-galloylshikimic acid (light) (IV) (adapted from Chen et al., 1997).

## ISOFLAVONOIDS AS PHYTOESTROGENS AND FLAVONOIDs AS ANTIESTROGENS

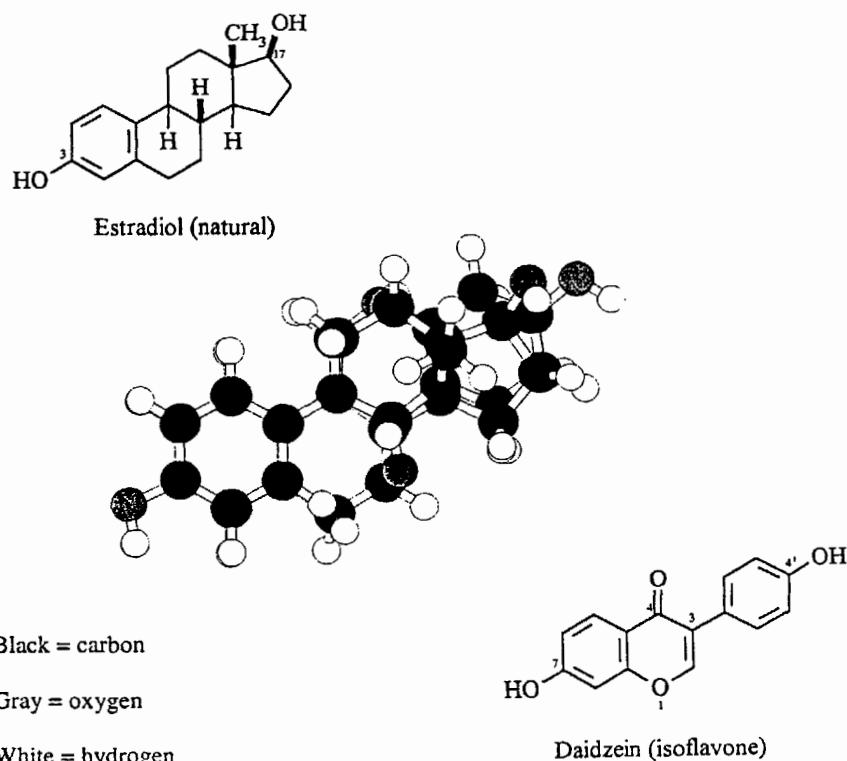
Many Chinese medicinal herbs are known to contain isoflavonoids. Lien's group (Lien et al., 1996; Lien and Lien, 1996) has reported that the estrogenic activities of many isoflavonoids can be attributed to their structural similarities with the natural estradiol and the synthetic diethylstilbestrol (see Figure 3). Among the physicochemical properties (Clog P,  $\mu$ , O-O distance, and MW) compared, the O-O distance appears to be within  $11 \pm 1\text{ \AA}$  for all the estrogenic isoflavonoids examined (Lien et al., 1996). Figure 4 shows the nearly perfect overlapping of daidzein and estradiol based on 3-D molecular modeling.

On the other hand, the structures of antiestrogenic flavonoids are quite different from those of isoflavonoids. From Figure 5, one can see that these antiestrogenic flavonoids have only one 4'-oxygen atom capable of H-bonding, but without the second 6-OH group equivalent to that in estradiol (Das et al., 1994). Another feature is that all antiestrogenic flavonoids have two additional OH or glycosylated OH groups at positions 5 and 7.

As shown in Figure 5, tamoxifen has structural similarity with flavonoids as shown in boldface, in spite of other structural differences. Tamoxifen has one *N,N*-dimethylaminoethoxy function on position 1 of the *cis* phenyl ring. SAR analysis indicates that 4'-hydroxytamoxifen, a minor metabolite of tamox-



**Figure 3** Structural similarity of estrogens and phytoestrogens (adapted from Lien and Lien, 1996).

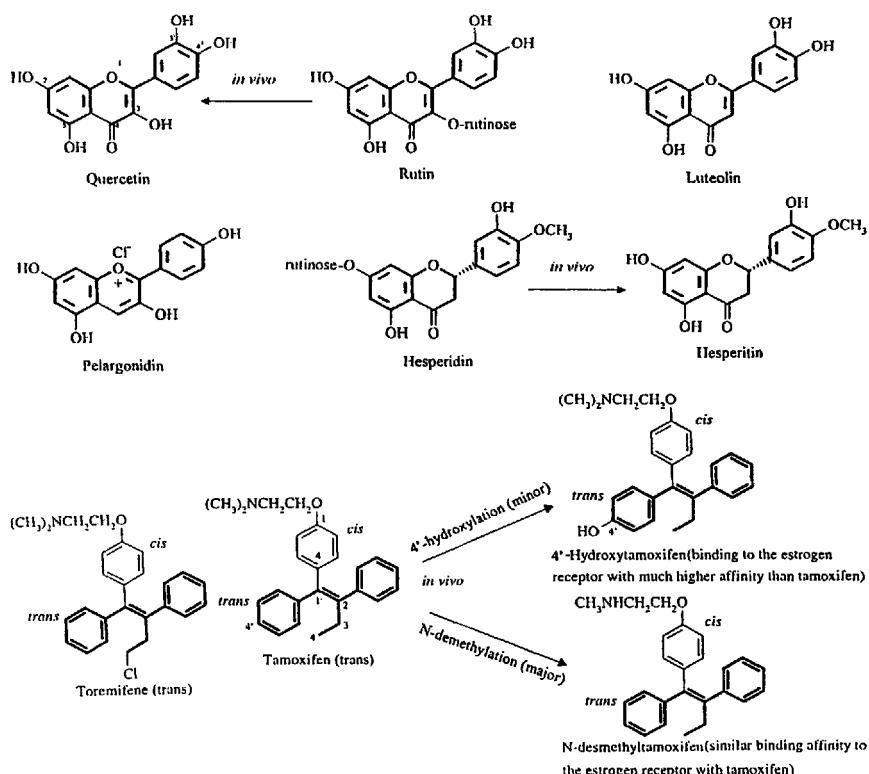


**Figure 4** Superimposed 3-D molecular modeling of natural estradiol and phytoestrogen daidzein, showing the nearly perfect overlap and very close O-O distance (adapted from Lien et al., 1996).

ifen, with an additional OH group on the *trans* phenyl ring has much higher binding affinity to the estrogen receptor. Furthermore, alterations in *N,N*-dimethylaminoethoxy side chain, even with an OH group, do not alter markedly the binding affinity to the estrogen receptor, suggesting that this part of the molecule may extend away from the actual binding site (Furr and Jordan, 1984). Toremifene, a new antiestrogen marketed in 1997, has one additional Cl attached to an ethyl side chain (Hussar, 1998). From Figure 5, one can see that tamoxifen and toremifene have the same backbone (highlighted in boldface for easier comparison) as antiestrogenic flavonoids.

## ANTIOXIDANT PHENOLICS—PHYSICOCHEMICAL PROPERTIES

Table 1 summarizes the physicochemical properties of different phenolic and other antioxidant compounds (see Figure 6 for the structures) according



**Figure 5** Structures of antiestrogenic flavonoids found in many plants, tamoxifen, and toremifene. Note the absence of two OH groups in these structures equivalent to those in estradiol and common backbones in these structures presented in boldface for easier comparison (adapted from Lien et al., 1996).

to the decreasing order of the lipophilic character (Clog P) for comparison. It is noteworthy that the calculated logarithm of octanol/water partition coefficient (Clog P) ranges over 17 log units, while the other parameters cover much narrower ranges. Due to the large ranges of hydrophobicity, it is likely that these phenols or their metabolites exert their biological effects in different tissue and cellular compartments. Further study has indicated that the Clog P values could be correlated with  $H_b$ , log MW, and  $\mu$ . This is in agreement with Lien's model published earlier (Lien, 1987).

$$\text{Clog P} = -0.308(0.221)H_b + 5.843(2.675) \quad (4)$$

$$n = 22$$

$$r^2 = 0.297$$

$$r = 0.545$$

$$s = 3.570$$

$$F_{t,20} = 8.43$$

$$p < 0.01$$

$$\text{Clog P} = -0.444(0.112)H_b + 15.118(3.856) \log \text{MW} - 28.555(8.867) \quad (5)$$

$$n = 22$$

$$r^2 = 0.845$$

$$r = 0.919$$

$$s = 1.718$$

$$F_{2,19} = 51.87$$

$$p < 0.0005$$

$$\begin{aligned} \text{Clog P} = & -0.443(0.117)H_b + 15.168(4.007) \log \text{MW} \\ & - 0.046(0.485)\mu - 28.588(9.142) \end{aligned} \quad (6)$$

$$n = 22$$

$$r^2 = 0.846$$

$$r = 0.920$$

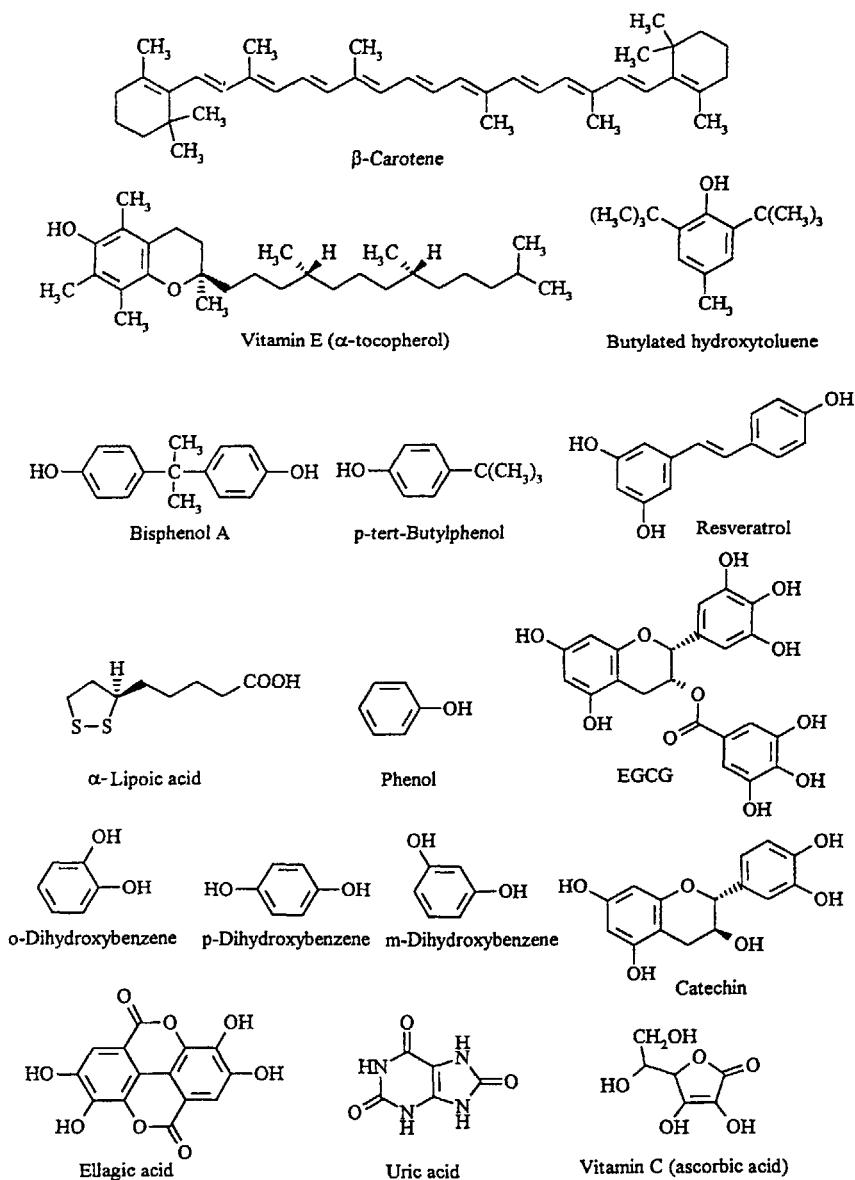
$$s = 1.764$$

$$F_{3,18} = 32.85$$

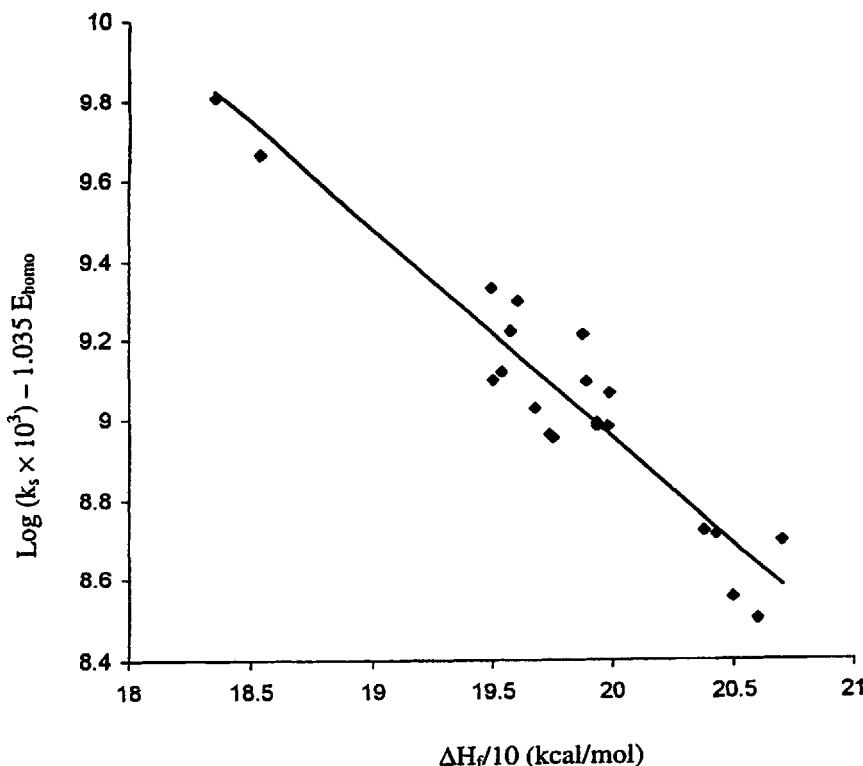
$$p < 0.0005$$

## **QSAR ANALYSIS OF THE ANTIOXIDANT ACTIVITIES OF VITAMIN E ANALOGS**

Vitamin E is the major chain-breaking antioxidant in body tissues and is considered the first line of defense against lipid peroxidation, protecting cell membranes at an early stage of free radical attack (Cadenas and Packer, 1996). It has been found that vitamin E has the protective role in preventing or minimizing free radical damage associated with cancer, cardiovascular disease, premature aging, cataracts, air pollution, and strenuous exercise (Cadenas and Packer, 1996). In order to estimate the antioxidant activities of new vitamin



**Figure 6** The chemical structures of different phenolic and other antioxidant compounds.



**Figure 7** A plot of  $\log (K_s \times 10^3)$  (after correcting for differences in  $E_{\text{homoe}}$ ) vs.  $\Delta H_f/10$ , showing  $\Delta H_f$  to be a good predictor of antioxidant activity of vitamin E analogs ( $n = 22$ ,  $r = 0.941$ ,  $s = 0.164$ ).

E analogs, QSAR analysis has been performed using calculated parameters (Lien et al., 1999). For 22 analogs, a significant correlation coefficient ( $r = 0.941$ ) was obtained.

$$\begin{aligned} \log (k_s \times 10^3) &= -0.516(0.251)\Delta H_f/10 \\ &\quad + 1.035(0.975)E_{\text{homoe}} + 19.290(4.680) \end{aligned} \quad (7)$$

$$n = 22$$

$$r^2 = 0.886$$

$$r = 0.941$$

$$s = 0.164$$

$$F_{2,20} = 73.78$$

$$p < 0.0005$$

As shown in Equation 7, a statistically significant correlation was found between the antioxidant activity [ $\log (k_s \times 10^3)$ ] and  $\Delta H_f$  (the difference of the heats of formation between radicals and the corresponding parent phenolic compounds) and  $E_{homo}$ . A plot of  $\log (k_s \times 10^3)$  values (after correcting for differences in  $E_{homo}$ ) vs.  $\Delta H_f$  is shown in Figure 7.

Electronic parameters that are often used in QSAR studies with vitamin E analogs are Hammett  $\sigma$  or Brown  $\sigma^+$  of the substituents attached to the phenol ring. Good correlations between the antioxidant properties ( $\log k_s$ ) and  $\Sigma\sigma$  or  $\Sigma\sigma^+$  were obtained when vitamin E analogs were split into one group with ( $n = 6$ ,  $r^2 = 0.878$ ) and one without ( $n = 10$ ,  $r^2 = 0.776$ ) the phytol side chain (van Acker et al., 1993). Another parameter used by Mukai et al. (1988) is the half peak oxidation potential ( $E_{p/2}$ ), which gives a good correlation with  $\log k_s$  ( $n = 13$ ,  $r^2 = 0.861$ ). However, because  $E_{p/2}$  has to be measured for every compound, it cannot be used to predict the antioxidant activity of new vitamin E analogs. This is clearly a disadvantage.

The correlation of antioxidant activities with  $\Delta H_f$  is not as good as that obtained with  $\Sigma\sigma^+$  or  $E_{p/2}$  (van Acker et al., 1993; Mukai et al., 1988), but  $\Delta H_f$  is relatively easy to calculate. Combination of  $\Delta H_f$  and other calculated parameters like  $E_{homo}$  is quite satisfactory for predicting the antioxidant activities of new vitamin E analogs. Furthermore, the correlation with these calculated parameters included a much more diverse group of vitamin E analogs, including those with different heterocyclic rings.

Several natural and synthetic vitamin E analogs have been compared for their biological activities based on rat assay by Bunyan et al. (Bunyan et al., 1961) and by Weiser and Vecchi (Weiser and Vecchi, 1982). It has been noted that the most critical chiral center appears to be position 2, and the least critical center is position 8' on the far end of the side chain, while the 4' position has moderate effect on the activity (Lien, 1995; Lien et al., 1999).

## CURCUMIN AND RELATED COMPOUNDS AS BLOCKERS OF SIGNAL TRANSDUCTION IN INHIBITION OF TUMOR PROMOTION

The rhizome of the plant *Curcuma longa* Linn. (Jiang Huang), commonly called turmeric, from the Zingiberaceae family, has been used for centuries as a spice and coloring agent in foods. The dry rhizome of turmeric contains demethoxycurcumin, bisdemethoxycurcumin, and curcumin. Curcumin is the main bioactive component, which also exists in other Chinese herbs like *Curcumia zedoaria*, *Curcuma aromatica*, and *Acorus calamus*. The chemical structures of these curcuminoids are shown in Figure 8. Curcumin has been shown to have remarkable antioxidant and free radical-scavenging (Kunchandy and Rao, 1990), anti-inflammatory (Mukhopadhyay et al., 1982; Satoskar et al., 1986), chemopreventive (Ren and Lien, 1997), and other biological

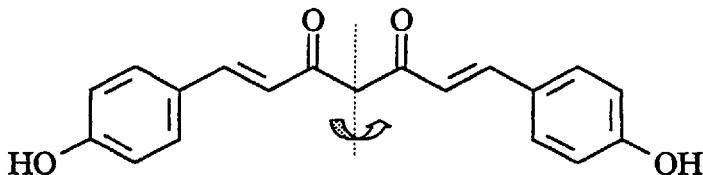
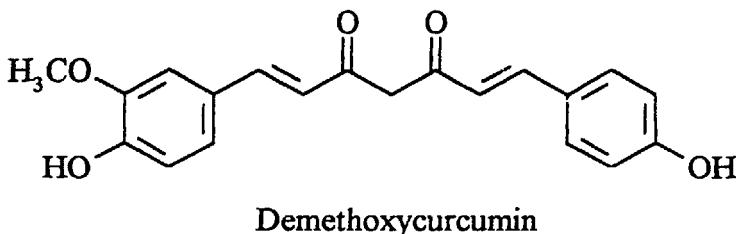
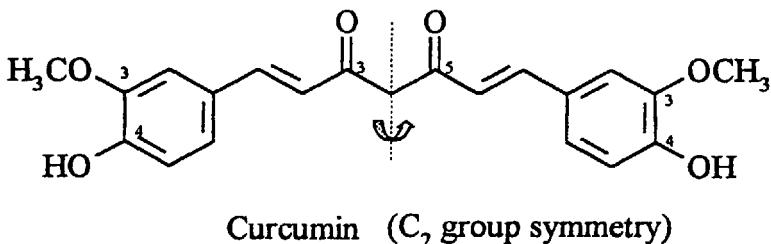
TABLE 1. Physicochemical properties of different phenolic and other antioxidant compounds in decreasing order of lipophilicity (clog P) (see figures 3–6 for the structures).

Category	Name	Clog P <sup>a</sup>	MW	H <sub>b</sub>	$\mu$ (D) <sup>b</sup>	$E_{\text{homo}}^{\text{b}}$ (eV)	$E_{\text{lumo}}^{\text{b}}$ (eV)	$E_{\text{T}}$ (V) <sup>c</sup>
Very lipophilic	$\beta$ -Carotene	15.69	536.88	0	0.02	-6.85	-1.64	0.72 <sup>d</sup>
	Vitamin E ( $\alpha$ -tocopherol)	12.28	430.71	5	6.32	-8.05	0.47	0.48 <sup>e</sup> (0.43) <sup>f</sup>
	Tamoxifen	6.64 (4.03 <sup>g</sup> )	371.52	3	1.40	-7.73	-0.83	
	Butylated hydroxytoluene (BHT)	5.23 (4.17 <sup>h</sup> )	220.35	3	1.31 (1.66 <sup>h</sup> )	-8.59	0.54	0.47 <sup>i</sup>
	Estradiol	3.78 (4.01 <sup>g</sup> )	272.39	6	2.43	-8.83	0.40	0.58 <sup>j</sup>
	Bisphenol A	3.67 (3.32 <sup>g</sup> )	228.29	6	1.07 (1.63 <sup>h</sup> )	-8.89	0.37	0.66 <sup>j</sup>
	p-tert-Butylphenol	3.30 (3.31 <sup>g</sup> )	150.22	3	1.26	-8.87	0.51	0.80 <sup>e</sup> (0.84) <sup>k</sup>
	Resveratrol	2.43	228.25	9	0.78	-8.14	-0.86	0.43 <sup>l</sup> (mono-OH) 0.69 <sup>l</sup> (di-OH)
	$\alpha$ -Lipoic acid	2.39	206.33	5	3.77	-8.26	-1.53	
	Curcumin	2.35 (4.26 <sup>g</sup> )	368.39	14	4.58	-8.96	-0.98	0.45 <sup>j</sup>
	Daidzein	1.58	254.24	10	1.86	-8.61	-0.65	
	Phenol	1.48 (1.47 <sup>g</sup> )	94.11	3	1.15 (1.51 <sup>h</sup> )	-9.04	0.36	0.97 <sup>e</sup> (0.96) <sup>k</sup>
	Epigallocatechin gallate (EGCG)	1.16	458.38	30	2.29	-9.22	-0.70	0.43 <sup>l</sup>
	Genistein	1.01	270.24	13	1.38	-8.81	-0.70	

TABLE 1. (continued).

Lipophilic	<i>o</i> -Dihydroxybenzene	0.88 (0.88 <sup>a</sup> )	110.11	6	1.98 (2.55 <sup>h</sup> )	-8.78	0.37	0.53 <sup>e</sup> (0.65)
	<i>p</i> -Dihydroxybenzene	0.81 (0.59 <sup>a</sup> )	110.11	6	0.00	-8.64	0.29	0.46 <sup>e</sup> (0.63 <sup>f</sup> )
	<i>m</i> -Dihydroxybenzene (resorcinol)	0.81 (0.80 <sup>a</sup> )	110.11	6	1.77 (1.99 <sup>h</sup> )	-8.91	0.36	0.81 <sup>e</sup> (0.82 <sup>f</sup> )
	Catechin	0.38	290.27	17	3.69	-8.72	0.21	0.57 <sup>g</sup>
	Ellagic acid	0.27	302.20	20	0.01	-9.50	-1.70	0.36 <sup>e</sup> (p-OH) 0.42 <sup>e</sup> (m-OH)
	Quercetin	0.13 (2.06 <sup>g</sup> )	302.24	19	1.14	-8.75	-1.20	0.33 <sup>g</sup>
	Uric acid	-1.46 (-2.92 <sup>c</sup> )	168.11	14	3.01	-8.94	-0.35	0.59
Hydrophilic	Vitamin C (ascorbic acid)	-2.21 (-1.64 <sup>g</sup> )	176.13	16	5.37	-9.76	-0.53	0.28 <sup>g</sup>

<sup>a</sup> Calculated logarithm of octanol/water partition coefficients (Clog P) using the CQSAR database (BioByte, 1998).<sup>b</sup> Calculated values using the HyperChem molecular modeling software (Hypercube, 1996).<sup>c</sup> Redox potentials at pH 7.<sup>d</sup> Measured value from Simic (1992).<sup>e</sup> Measured values from Lien et al. (1998).<sup>f</sup> Calculated values from Lien et al. (1999, Equation 6).<sup>g</sup> Measured log P values from the CQSAR database.<sup>h</sup> Measured dipole moment values from McClellan (1989).<sup>i</sup> Measured values from Rice-Evans and Packer (1998).<sup>j</sup> Measured values from Jovanovic et al. (1991).



Bisdemethoxycurcumin ( $C_2$  group symmetry)

**Figure 8** The chemical structures of curcuminoids.

activities (Ammon and Wahl, 1990; Srivastava et al., 1985). The antitumor-promotion effects of curcumin in different model systems are summarized in Table 2.

The molecular mechanisms of the antitumor-promotion activity of curcumin have been investigated by various investigators. Curcumin acts as a chemopreventive agent for inhibiting tumor promotion based on the following signal transduction pathways (Table 3 and Figure 9 for the target sites).

Curcumin inhibits DNA synthesis by inhibition of thymidine kinase (TK) (Singh et al., 1996) and thymidine incorporation into DNA (site 1, Figure 9) (Huang et al., 1988). It inhibits transcription by suppression of c-Jun mRNA (site 2) (Huang et al., 1991) and c-Jun N-terminal kinase (JNK) pathway (site

TABLE 2. Chemopreventive effects of curcumin in various model systems.

Organ	Animal	Carcinogen	Dose Regimens of Curcumin	Reference
Skin	CD-1 mice	TPA-induced/DMBA-initiated	1-10 $\mu$ mol, twice/w, 20 ws.	Huang et al., 1988
	Swiss mice	DMBA-initiated	200 nmol, twice/w, 3 ws.	Nagabhushan and Bhide, 1992
	Swiss mice	DMBA-initiated/TPA-promoted	200 nmol, twice/w, 12 ws.	Nagabhushan and Bhide, 1992
	Swiss mice	B( $\alpha$ )P-induced	1 mg/mouse, 4 ws.	Nagabhushan and Bhide, 1992
	A/J mice	B( $\alpha$ )P-induced	0.5-2% in the diet, 7 ws.	Huang et al., 1994
Forestomach	rat	DMBA-induced hyperplastic nodules	1 $\mu$ M	Mehta and Moon, 1991
Mammary	Wistar rat	DMBA-induced	5.1 mg/day/mouse	Bhide et al., 1994
	Sprague-Dawley rat	DMBA-induced	100 or 200 mg/kg, i.p., once/d, 5 ds.	Singletary et al., 1996
	F344 rat	AOM-induced crypts	2000 ppm in the diet, 2 ws.	Rao et al., 1993
Colon	CF-1 mice	AOM-induced	0.5-4% in the diet	Huang et al., 1994
Oral mucosa	Syrian golden hamsters	MAMNA-induced	5% in the diet, 2 ws.	Azuine and Bhide, 1994
Tongue	F344 rat	NQO-initiated	0.5 g/kg in the diet	Tanaka et al., 1994.
Duodenum	C57BL/6 mice	ENNG-induced	0.5-2% in the diet	Huang et al., 1994

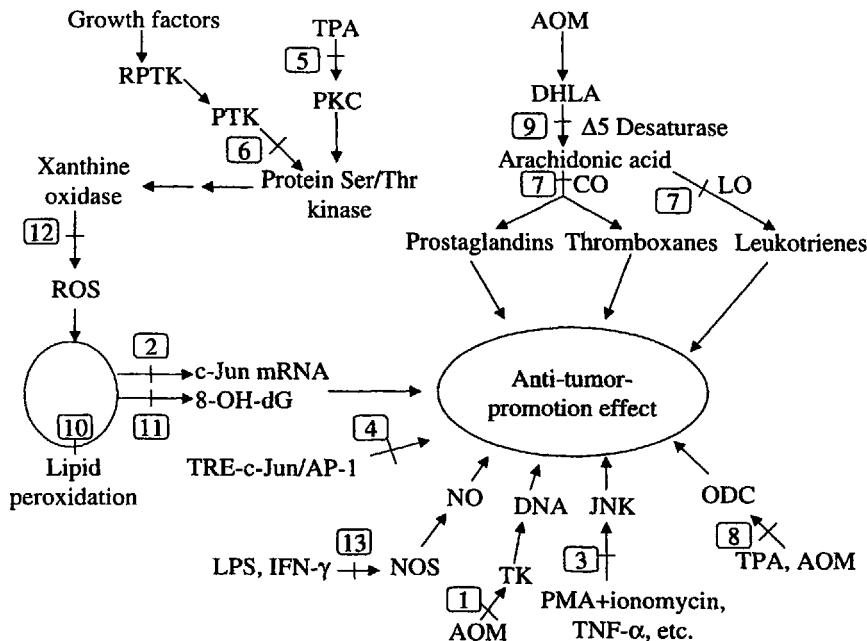
Abbreviations: AOM: azoxymethane; B( $\alpha$ )P: benzo( $\alpha$ )pyrene; DMBA: 7,12-dimethylbenz( $\alpha$ )anthracene; ENNG: N-ethyl-N'-nitro-nitrosoguanidine; MAMNA: methyl-(a oxymethyl)-nitrosamine; NQO: 4-nitroquinoline-N-oxide; TPA: 12-O-tetradecanoylphorbol-13-acetate; w = weeks; ws = weeks; d = day; ds = days.

## 36 QSAR AND MOLECULAR MODELING OF BIOACTIVE PHYTO-PHENOLICS

TABLE 3. Different molecular mechanisms of anti tumor-promotion activity of curcumin proposed by various investigators.

Mechanisms	Sites of Action	Dose Regimens of Curcumin	Reference
Inhibition of DNA synthesis	Thymidine kinase (site 1)	1–10 $\mu\text{M}$	Singh et al., 1996
	Thymidine incorporation into DNA (site 1)		Huang et al., 1988
Inhibition of transcription	c-Jun mRNA (site 2)	10–20 $\mu\text{M}$	Huang et al., 1991
	c-Jun N-terminal kinase (JNK) pathway (site 3)	$\text{IC}_{50} = 5\text{--}10 \mu\text{M}$	Chen and Tan, 1998
Inhibition of translation	TPA-responsive element (TRE) binding by c-Jun/AP-1 protein (site 4)	$\text{IC}_{50} < 20 \mu\text{M}$	Huang et al., 1991
Inhibition of enzymes	Protein kinase C (PKC) (site 5)	15–20 $\mu\text{M}$	Lin et al., 1997; Liu et al., 1993
	Tyrosine kinase (site 6)	2000 ppm	Rao et al., 1993
	Cylooxygenase and lipoxygenase (site 7)	$\text{IC}_{50} = 5\text{--}10 \mu\text{M}$	Huang et al., 1991, 1997
	Ornithine decarboxylase (ODC) (site 8)	0.5–10 $\mu\text{mol}$ or 1–10 $\mu\text{M}$ or 2000 ppm	Huang et al., 1988; Lu et al., 1993; Rao et al., 1993
	$\Delta 5$ desaturase (site 9)	$\text{IC}_{50} = 27.2 \mu\text{M}$	Shimizu et al., 1992
Free radical scavenger	Lipid peroxidation (site 10)	5–10 $\mu\text{M}$	Shih and Lin, 1993
	8-Hydroxydeoxyguanosine (8-OH-dG) (site 11)	5–10 $\mu\text{M}$	Shih and Lin, 1993
	Xanthine oxidase (site 12)	2–10 $\mu\text{M}$	Lin and Shih, 1994
	Nitric oxide synthase (NOS) (site 13)	$\text{IC}_{50} < 1 \text{ mM}$	Chan et al., 1998; Soliman and Mazzio, 1998

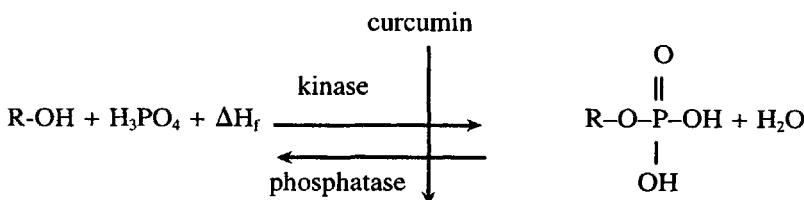
3) (Chen and Tan, 1998). It also inhibits translation by inhibition of TPA-responsive element (TRE) binding by c-Jun/AP-1 protein (site 4) (Huang et al., 1991). Curcumin has also been shown to inhibit various enzymes including protein kinase C (PKC) (site 5) (Lin et al., 1997; Liu et al., 1993; Rao et al., 1993), tyrosine kinase (site 6) (Stoner and Mukhtar, 1995), cyclooxygenase and lipoxygenase (site 7) (Huang et al., 1991, 1997), ornithine decarboxylase (ODC) (site 8) (Huang et al., 1988; Lu et al., 1993; Rao et al., 1993), and  $\Delta 5$  desaturase (site 9) (Shimizu et al., 1992). Curcumin also scavenges free radicals to inhibit lipid peroxidation (site 10) and 8-hydroxydeoxyguanosine (8-OH-



**Figure 9** Multiple target sites of the signal transduction pathway by curcumin (see text for the target sites). AOM: azoxymethane; AP-1: activator protein; CO: cyclooxygenase; DHLA: dihomoy-linolenic acid; IFN- $\gamma$ : interferon- $\gamma$ ; JNK: c-Jun N-terminal kinase; LO: lipoxygenase; LPS: lipopolysaccharide; NO: nitric oxide; NOS: nitric oxide synthase; ODC: ornithine decarboxylase; 8-OH-dG: 8-hydroxydeoxyguanosine; PKC: protein kinase C; PMA: phorbol 12-myristate 13-acetate; PTK: protein tyrosine kinase; ROS: reactive oxygen species; RPTK: receptor protein tyrosine kinase; TK: thymidine kinase; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; TPA: 12-O-tetradecanoylphorbol-13-acetate; TRE: TPA-responsive element.

dG) (site **11**) formation (Shih and Lin, 1993), and directly inactivates xanthine oxidase to reduce superoxide generation (site **12**) (Lin and Shih, 1994) and nitric oxide synthase (NOS) to reduce nitric oxide (NO) production (site **13**) (Chan et al., 1998; Soliman and Mazzio, 1998).

From the mechanisms of action of curcumin reported, it appears that one common reaction is involved in the kinase-catalyzed signal transduction pathway, namely the endothermic dehydration step:



where R-OH can be tyrosine, serine, or threonine. These reactions are endothermic with a  $\Delta H_f$  of 17.87, 14.20, and 14.69 kcal/mol for tyrosine, serine, and threonine, respectively, indicating that the phosphorylation step is energy costing. This finding is consistent with the results in our previous report (Lien and Ren, 1998). Another common reaction seems to be free radical-mediated reaction. Phenolics enter the reaction process either by free radical reaction or preferential oxidation. Chemical reactions in which curcumin and other phenolics are involved in the crosstalks among the different pathways have not been fully understood. Further investigations are needed to delineate these interactions.

## CONCLUSION

In summary, the inhibitory activities of lignans and tannins against HIV-1 RT and hDNAP- $\alpha$  have been successfully correlated with  $\mu$ , log MW,  $H_b$ , and I. Furthermore, 3-D structures of the most active compounds reveal that there are different structural requirements for differential inhibition of HIV-1 RT and hDNAP- $\alpha$ . Structural similarities of estrogenic isoflavonoids with the natural estradiol and the synthetic diethylstilbestrol, and antiestrogenic flavonoids with the synthetic antiestrogenic tamoxifen and toremifene are compared. The heat of formation and other calculated physicochemical parameters are shown to be useful in predicting the antioxidant activities of vitamin E analogs. Curcumin, as an inhibitor of DNA synthesis, transcription, translation, and different enzymes involved in signal transduction and free radical formation, has been found to be an effective chemopreventive agent against different chemical carcinogens.

Due to the inherent diversity in phytochemicals evolved through the ages, only a very small percentage has been studied. They will continue to provide leads to new drug discovery and development. By systematic investigation, it is hoped that many new applications can be found in old remedies and new plants.

## ACKNOWLEDGEMENT

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# **Chemoprevention by Phytochemical Modifiers of Carcinogen Metabolism**

**STEPHEN S. HECHT**

## **INTRODUCTION**

A variety of carcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic aromatic amines, *N*-nitroso compounds, and aflatoxins, are believed to be causes of major human cancers (Baird and Ralston, 1997; Delelos and Kadlubar, 1997; Adamson et al., 1995; Hecht, 1998a; Kensler and Groopman, 1997). Virtually all carcinogens to which humans are exposed require enzymatic transformation to exert their carcinogenic effects. The most common enzymatic process is addition of oxygen, catalyzed by cytochrome P450 enzymes (Guengerich, 1997). This generally increases the polarity of the molecule facilitating excretion. This type of transformation is referred to as Phase 1 metabolism. Some of the intermediates formed in this process may be electrophiles, which can react with nucleophilic sites in critical macromolecules, such as DNA, RNA, and protein. The DNA adducts that are formed can persist if they escape cellular repair mechanisms. These adducts have the potential to cause miscoding, thus producing permanent mutations in critical genes, such as oncogenes and tumor suppressor genes (Bowden, 1997; Balmain, 1997). Multiple mutations of this type are involved in cancer induction. The conversion of a carcinogen to a macromolecular adduct is called metabolic activation. Competing with metabolic activation is detoxification. A second group of enzymes, Phase 2 enzymes, are important in detoxification. These enzymes, typified by glutathione-S-transferases, UDP-glucuronosyl transferases, and sulfotransferases, add polar moieties to the oxygenated carcinogen, generally producing highly polar molecules that are readily excreted (Armstrong, 1997; Burchell et al., 1997; Duffel, 1997).

Blocking carcinogen metabolic activation or enhancing detoxification are ways to decrease carcinogenicity. A large number of compounds found in edible plants have these properties and are variously known as anticarcinogens, cancer chemopreventive or chemoprotective agents. Among these, sulfur-containing compounds have been studied extensively. Reviews by Wattenberg in 1978 already cited numerous examples of chemoprevention by these compounds (Wattenberg, 1978a, b). Recent reviews extensively document their chemopreventive activities and discuss relevant mechanisms (Stoewsand, 1995; Verhoeven et al., 1997; Johnson et al., 1994; Jongen, 1996; Smith and Yang, 1994; Hecht, 1995; Wattenberg, 1992; Wargovich, 1992; Lea, 1996). Another widely studied compound, indole-3-carbinol, has mixed activities. In some systems, it is a chemopreventive agent, while in others it can promote tumorigenesis (Dashwood, 1998). This chapter will discuss isothiocyanates and indole-3-carbinol, which are derived from glucosinolates that occur in vegetables of the family Cruciferae, and thiols present in genus *Allium* plants. The discussion will be limited mainly to naturally occurring compounds and will attempt to provide representative examples of chemopreventive activities and mechanisms.

## ISOTHIOCYANATES AND GLUCOSINOLATES

### OCCURRENCE AND FORMATION

Isothiocyanates occur in plants as thioglucoside conjugates called glucosinolates (Fenwick et al., 1989). More than 100 glucosinolates have been identified, mainly in vegetables of the family Cruciferae (Verhoeven et al., 1997; Fenwick et al., 1989; Tookey et al., 1980). Common vegetables of this family are summarized in Table 1 (Verhoeven et al., 1997). Hydrolysis of the glucosinolates is catalyzed by multiple forms of the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which occur in the same plants, separated cellularly from the glucosinolates. When the plant is macerated or chewed, myrosinase mixes with the glucosinolate and affects the hydrolysis as illustrated in Figure 1. Myrosinase activity has also been found in some intestinal microflora, which is important with respect to intake of intact glucosinolates (Verhoeven et al., 1997). Myrosinase catalyzes hydrolysis of the glucosinolate S-sugar bond leading to an unstable thiohydroxamic acid that undergoes a Lossen rearrangement yielding the isothiocyanate. Depending on the nature of the R group and the conditions, other products such as nitriles and thiocyanates may also form.

A large number of glucosinolates with many different R groups occur in substantial quantities in cruciferous plants and crops. This area has been extensively reviewed (Fenwick et al., 1989; Tookey et al., 1980). Typical

TABLE 1. Common vegetables of the family cruciferae.

Genus	Species	Common Name
<i>Armoracia</i>	<i>rusticana</i>	Horseradish
	<i>campestris</i>	Turnip
	<i>chinensis</i>	Pak choy
	<i>juncea</i>	Brown mustard
	<i>napus</i>	Rape, swede, rutabaga
	<i>nigra</i>	Black mustard
	<i>oleracea</i>	Cabbage, kale, brussels sprouts, cauliflower, broccoli, kohlrabi
<i>Lepidium</i>	<i>pekinensis</i>	Chinese cabbage
<i>Nasturtium</i>	<i>sativum</i>	Garden cress
<i>Raphanus</i>	<i>officinale</i>	Watercress
<i>Sinapis</i>	<i>sativus</i>	Radish
	<i>alba</i>	Mustard

From Verhoeven et al., 1997.

glucosinolate contents in agriculturally important plants such as cabbage, brussels sprouts, cauliflower, turnip, radish, and watercress range from approximately 0.5 to 3 mg/g of fresh plant materials (Tookey et al., 1980).

## INHIBITION OF CARCINOGENESIS BY ISOTHIOCYANATES, GLUCOSINOLATES, AND CRUCIFEROUS VEGETABLES

Studies on inhibition of carcinogenesis by isothiocyanates are summarized in Table 2. A wide variety of isothiocyanates, both naturally occurring and synthetic, have been tested. Naturally occurring isothiocyanates with chemopreventive activity include benzyl ( $R = PhCH_2$ , BITC), 2-phenylethyl ( $R = PhCH_2CH_2$ , PEITC), 3-phenylpropyl ( $R = PhCH_2CH_2CH_2$ , PPITC), and sulforaphane ( $R = CH_3S(O)(CH_2)_4$ ). Among these, BITC and PEITC are the most extensively studied. BITC is an effective inhibitor of rat mammary and mouse lung tumorigenesis by the polycyclic hydrocarbons DMBA (7,12-dimethylbenz[a]anthracene) and BaP (benzo[a]pyrene). It is less effective in nitrosamine-

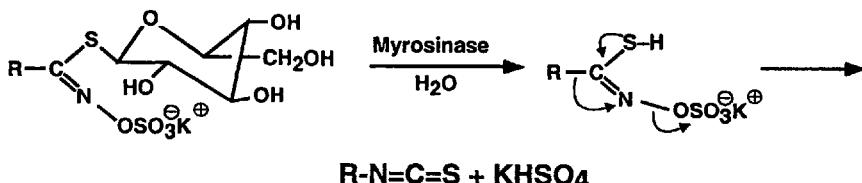


Figure 1 Formation of isothiocyanates in the myrosinase catalyzed hydrolysis of glucosinolates.

TABLE 2. Modification of carcinogenesis by isothiocyanates.

Isothiocyanate R-N=C=S; R =	Naturally Occurring <sup>a</sup>	Carcinogen <sup>b</sup>	Species and Target Organ	Effect	Reference
$\alpha$ -Naphthyl-	No	3'-Me-DAB	Rat liver	Inhibition	Sasaki, 1963
		Ethionine	Rat liver	Inhibition	Sidransky et al., 1966
		AAF	Rat liver	Inhibition	Sidransky et al., 1966
		DAB	Rat liver	Inhibition	Lacassagne et al., 1970
		<i>m</i> -Toluylenediamine	Rat liver	Inhibition	Ito et al., 1969
	Yes	NDEA	Rat liver	No effect	Makiura et al., 1973
		BHBN	Rat bladder	Inhibition	Ito et al., 1974
		DAB	Rat liver	Inhibition	Lacassagne et al., 1970
		DMBA	Rat mammary	Inhibition	Wattenberg, 1977
		NNK	Mouse lung	No effect	Morse et al., 1989a
$\beta$ -Naphthyl- Ph-	Yes	DMBA	Rat mammary	Inhibition	Wattenberg, 1977, 1981
			Mouse forestomach	Inhibition	Wattenberg, 1977
			Mouse lung	Inhibition	Wattenberg, 1977
		BaP	Mouse lung	Inhibition	Lin et al., 1993; Wattenberg, 1987
			Mouse forestomach	Inhibition or no effect	Lin et al., 1993; Wattenberg, 1987
	Yes	NNK	Mouse skin	No effect	Lin et al., 1993
		NDEA	Mouse lung	No effect	Morse et al., 1989a, 1990b
			Mouse forestomach	Inhibition	Wattenberg, 1987
			Mouse lung	No effect	Wattenberg, 1987
		MAM	Rat liver	Inhibition	Sugie et al., 1993
$\text{PhCH}_2$	Yes		Rat small intestine/ colon	Inhibition	Sugie et al., 1994
		NBMA	Rat esophagus	No effect	Wilkinson et al., 1995
		NDEA + BHBN	Rat bladder	Enhancement	Hirose et al., 1998

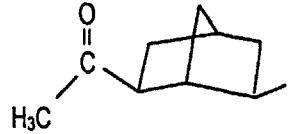
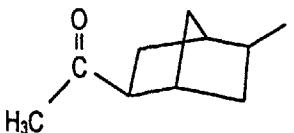
TABLE 2. (*continued*).

$\text{Ph}(\text{CH}_2)_2\cdot$	Yes	DMBA	Rat mammary	Inhibition or no effect	Wattenberg, 1977; Lubet et al., 1997; Futakuchi et al., 1998
			Mouse forestomach	Inhibition	Wattenberg, 1977
			Mouse lung	Inhibition	Wattenberg, 1977
		NNK	Rat lung	Inhibition	Chung et al., 1996; Hecht et al., 1996b; Morse et al., 1989c
			Rat nasal cavity, liver	No effect	Morse et al., 1989c
			Mouse lung	Inhibition	Morse et al., 1989a, b, 1991, 1992; Matzinger et al., 1995; El-Bayoumy et al., 1996; Jiao et al., 1997
		NDEA	Mouse lung	No effect	Morse et al., 1990b
		NBMA	Mouse liver	Inhibition	Pereira, 1995
			Rat esophagus	Inhibition or no effect	Siglin et al., 1995; Stoner et al., 1991; Wilkinson et al., 1995
		BOP	Hamster pancreas and lung	Inhibition	Nishikawa et al., 1996b
$\text{Ph}(\text{CH}_2)_3\cdot$	Yes	BaP	Mouse lung	No effect	Adam-Rodwell et al., 1993; Lin et al., 1993
			Mouse skin	No effect	Lin et al., 1993
		NDEA + BHBN	Rat bladder	Enhancement	Hirose et al., 1998
		NNK	Mouse lung	Inhibition	Morse et al., 1989b, 1991
		NBMA	Rat esophagus	Inhibition	Wilkinson et al., 1995
$\text{Ph}(\text{CH}_2)_4\cdot$	Yes	BOP	Hamster lung	Inhibition	Nishikawa et al., 1996a
		NNN	Rat esophagus	Inhibition	Stoner et al., 1998
$\text{Ph}(\text{CH}_2)_5\cdot$	Yes	NNK	Mouse lung	Inhibition	Morse et al., 1989b, 1991
		NBMA	Rat esophagus	Inhibition	Wilkinson et al., 1995
$\text{Ph}(\text{CH}_2)_6\cdot$	No	NNK	Mouse lung	Inhibition	Morse et al., 1991
		NNK	Mouse lung	Inhibition	Morse et al., 1991, 1992; Jiao et al., 1997

TABLE 2. (*continued*).

Isothiocyanate R-N = C = S; R =	Naturally Occurring <sup>a</sup>	Carcinogen <sup>b</sup>	Species and Target Organ	Effect	Reference
			Mouse skin	No effect	Lin et al., 1993
			Rat lung	Inhibition	Chung et al., 1996; Hecht et al., 1996a
		NBMA	Rat esophagus	Enhancement	Stoner et al., 1995
		AOM	Rat colon	Enhancement	Rao et al., 1995
Ph(CH <sub>2</sub> ) <sub>8</sub> -	No	NNK	Mouse lung	Inhibition	Jiao et al., 1994
Ph(CH <sub>2</sub> ) <sub>10</sub> -	No	NNK	Mouse lung	Inhibition	Jiao et al., 1994
PhCH(Ph)CH <sub>2</sub> -	No	NNK	Mouse lung	Inhibition	Jiao et al., 1994
PhCH <sub>2</sub> CH(Ph)-	No	NNK	Mouse lung	Inhibition	Jiao et al., 1994
CH <sub>2</sub> =CHCH <sub>2</sub> -	Yes	NNK	Mouse lung	No effect	Jiao et al., 1994
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>5</sub> -	Yes	NNK	Mouse lung	Inhibition	Jiao et al., 1994
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> )-	?	NNK	Mouse lung	Inhibition	Jiao et al., 1994
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>11</sub> -	No	NNK	Mouse lung	Inhibition	Jiao et al., 1994, 1996
3-PyrC(CH <sub>2</sub> ) <sub>3</sub> -    O	No	NNK	Mouse lung	No effect	Morse et al., 1989b
9-Phenanthryl-	No	BaP	Mouse skin	No effect	Lin et al., 1993
9-Methylenephенanthryl-	No	BaP	Mouse skin	No effect	Lin et al., 1993
6-Chrysanyl-	No	BaP	Mouse skin	No effect	Lin et al., 1993
6-Benzo[a]pyrenyl-	No	BaP	Mouse skin	No effect	Lin et al., 1993
CH <sub>3</sub> S(CH <sub>2</sub> ) <sub>4</sub> -    O	Yes	DMBA	Rat mammary	Inhibition	Zhang et al., 1994

TABLE 2. (continued).

	No	DMBA	Rat mammary	Inhibition	Zhang et al., 1994
	No	DMBA	Rat mammary	Inhibition	Zhang et al., 1994
	No	DMBA	Rat mammary	Inhibition	Zhang et al., 1994

<sup>a</sup> Based on Fenwick et al., 1989.

<sup>b</sup> Abbreviations: AOM, azoxymethane; BOP, *N*-nitrosobis(2-oxopropyl)amine; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene; AAF, 2-acetylaminofluorene; DMBA, 7,12-dimethylbenz [a]anthracene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; BaP, benzo[a]pyrene; NDEA, *N*-nitroso-diethylamine; MAM, methylazoxymethanol acetate; NBMA, *N*-nitrosobenzylmethylamine; BHBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.

induced tumor models. In contrast, PEITC has broad inhibitory activity against tumors induced by nitrosamines. This includes inhibition of lung tumorigenesis in mice and rats by the tobacco-specific carcinogen NNK [4-methylnitrosamo-1-(3-pyridyl)-1-butanone], inhibition of liver tumor induction by NDEA (*N*-nitrosodiethylamine) in the mouse, inhibition of esophageal tumor induction by NBMA (*N*-nitrosobenzyl methylamine) in the rat, and inhibition of pancreas and lung tumorigenesis by BOP in the hamster. Inhibition of NNK-induced pulmonary carcinogenesis by PEITC has been demonstrated in multiple studies in mice and rats; this compound is presently in Phase I clinical trials in healthy smokers (National Cancer Institute, 1996b). Structure-activity studies demonstrate that increased isothiocyanate lipophilicity increases inhibitory potency (Jiao et al., 1994). Thus, single doses of 10-phenyldecy1 isothiocyanate or 1-dodecyl isothiocyanate as low as 0.04 to 1  $\mu\text{mol}$  are sufficient to inhibit mouse lung tumorigenesis induced by a single dose of 10  $\mu\text{mol}$  NNK (Jiao et al., 1994). Further studies demonstrate that the isothiocyanate group, but not the phenyl ring, is necessary for inhibition and that lower reactivity with glutathione leads to better inhibitory potency (Jiao et al., 1994; Jiao et al., 1996). *N*-Acetylcysteine and glutathione conjugates of PEITC also show inhibitory activity against mouse lung tumorigenesis by NNK (Jiao et al., 1997). While PEITC is a superb inhibitor of nitrosamine-induced carcinogenicity in multiple tumor models, it is less effective against polycyclic aromatic hydrocarbons (PAH). Bioassays carried out to date fail to demonstrate inhibition of BaP-induced mouse lung or skin tumorigenesis by PEITC. Mixed results have been obtained in the DMBA rat mammary tumor model. Initial studies by Wattenberg, in which PEITC was given by gavage, showed inhibition of mammary tumorigenesis (Wattenberg, 1977). A recent study by Lubet et al. in which PEITC was given in the diet showed no effect or somewhat enhanced mammary tumorigenesis by DMBA (Lubet et al., 1997). However, another recent dietary study demonstrated that carcinoma volume, but not multiplicity or incidence, was decreased by PEITC (Futakuchi et al., 1998). The effects of PEITC on carcinogenesis by PAH require further study.

The studies summarized in Table 2 demonstrate inhibition of carcinogenesis mainly when isothiocyanates are given either before or before and during carcinogen administration. Few studies demonstrate inhibition by isothiocyanates given after carcinogen treatment, although BITC does inhibit DMBA-induced mammary carcinogenesis when administered in this way (Wattenberg, 1981). For reasons discussed below, it may be important to investigate further the ability of isothiocyanates to inhibit carcinogenesis when given in the post-initiation phase.

Enhancement of tumorigenesis has been observed in some studies with isothiocyanates. Both BITC and PEITC promote urinary bladder carcinogenesis in rats treated with NDEA and BHBN [*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine], although the dose employed was higher than that used for chemoprevention (Hirose et al., 1998). 6-Phenylhexyl isothiocyanate ( $R = \text{Ph}(\text{CH}_2)_6$ , PHITC), which is not known to be naturally occurring, enhances colon carci-

genesis and esophageal carcinogenesis in rat tumor models (Stoner et al., 1995; Rao et al., 1995).

Relatively few studies have been carried out on the effects of glucosinolates on carcinogenesis, probably because the compounds are generally less available in pure form. These studies have been reviewed recently (Verhoeven et al., 1997). Sinigrin, the glucosinolate with R = allyl, inhibits liver and tongue tumors in rat models, but has no effect on lung, liver, or nasal tumors induced by NNK (Verhoeven et al., 1997; Morse et al., 1988). Sinigrin may enhance pancreatic tumorigenesis in NNK-treated rats (Morse et al., 1988). Sinigrin also inhibits DMH-induced aberrant crypt foci and induces apoptosis in rat colon (Smith et al., 1998). Glucobrassicin, the precursor to indole-3-carbinol, inhibits BaP-induced lung and forestomach tumors in mice, while glucotropaeolin (R = benzyl) and glucosalbin (R = 4-hydroxybenzyl) have little or no effect (Wattenberg et al., 1986). Glucobrassicin and glucotropaeolin inhibit DMBA-induced mammary tumors in the rat (Wattenberg et al., 1986). It should be noted that glucosinolates also have well-documented toxic effects, particularly goitrogenicity (Fenwick et al., 1989; Tookey et al., 1980; McDanell et al., 1988).

A modest number of studies have investigated the effects of cruciferous vegetables on tumorigenesis; these have been reviewed (Stoewsand et al., 1995; Verhoeven et al., 1997; McDanell et al., 1988). Several studies show that cabbage or cauliflower decrease tumor formation in rat and mouse models; however, enhancement of pancreatic and skin tumorigenesis has been observed in cabbage-fed hamsters and mice (Birt et al., 1987). A recent investigation demonstrates protective effects of cruciferous seed meals and hulls against colon cancer in mice (Barrett et al., 1998). The complexity of vegetables prevents direct assignment of their inhibitory properties to particular constituents. However, based on studies carried out to date, it is plausible that isothiocyanates and other hydrolysis products of glucosinolates in vegetables are at least partially responsible for inhibition of carcinogenesis by vegetables.

## MECHANISMS OF CHEMOPREVENTION BY ISOTHIOCYANATES

Isothiocyanates can profoundly affect carcinogen metabolism. Numerous studies demonstrate that isothiocyanates inhibit specific cytochrome P450 enzymes involved in the activation and detoxification of carcinogens. Other studies show that isothiocyanates induce Phase 2 enzymes such as glutathione-S-transferases and quinone reductases. These studies have been reviewed (Smith and Yang, 1994; Zhang and Talalay, 1994; Yang et al., 1994). While many studies have investigated the effects of isothiocyanates on these enzymes, fewer have looked at the effects of isothiocyanates on carcinogen metabolism in the specific models where inhibition of tumor development has been observed. For example, sulforaphane is known to be a potent inducer of glutathione-S-transferases and quinone reductases, but there is no evidence that induc-

tion of these enzymes is specifically responsible for its inhibitory effects on DMBA-induced mammary tumorigenesis (Zhang et al., 1994; Zhang et al., 1992). Moreover, sulforaphane also inhibits cytochrome P450 activity (Maheo et al., 1997). PEITC is the most extensively studied chemopreventive isothiocyanate with respect to mechanisms of inhibition of rat lung tumorigenesis by NNK and rat esophageal tumorigenesis by NBMA.

Studies on inhibition of NNK-induced rat lung carcinogenesis by PEITC clearly show that its major effect is specific inhibition of cytochrome P450 enzymes in the rat lung, which are responsible for the metabolic activation of NNK. In studies carried out under the conditions of the bioassay in which PEITC inhibited rat lung tumorigenesis by NNK, we demonstrated that PEITC had no effect on the distribution of NNK and its metabolites in different tissues of the rat, although levels of metabolites resulting from the metabolic activation of NNK were reduced in the lung of PEITC-treated rats (Staretz and Hecht, 1995). We also showed that while PEITC had no effect on hepatic microsomal metabolism of NNK and its major metabolite NNAL, it specifically inhibited the metabolic activation of both NNK and NNAL [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol] in the rat lung (Staretz et al., 1997b). In a third study, we examined the effects of PEITC on DNA adducts of NNK in the rat lung and in individual cell types of the lung (Staretz et al., 1997a). The results demonstrated that PEITC significantly inhibited DNA pyridyloxobutylation by NNK, particularly in the Type II cells, which are the targets of rat lung tumorigenesis by NNK.

Extensive studies also demonstrate that inhibition of cytochrome P450s by isothiocyanates is the major mechanism by which they inhibit NNK-induced lung tumorigenesis in the mouse (Hecht, 1998b; Guo et al., 1993; Smith et al., 1990, 1993). This results in inhibition of O<sup>6</sup>-methylguanine formation and tumorigenesis (Morse et al., 1989a, 1991). When added to microsomal incubations, PEITC inhibits NNK oxidation by competitive and noncompetitive mechanisms (Smith et al., 1990, 1993). Longer chain arylalkyl isothiocyanates are stronger inhibitors of NNK metabolic activation than is PEITC, which correlates with the tumor inhibition data (Guo et al., 1993; Smith et al., 1993). For example, PHITC is a potent competitive inhibitor of NNK oxidation in mouse lung microsomes with an apparent K<sub>i</sub> of 11 to 16 nM (Guo et al., 1993). Dietary PEITC has significant effects on cytochrome P450 enzymes in the mouse, but little effect on Phase 2 enzymes (Guo et al., 1993; Smith et al., 1993). In the mouse, dietary PEITC and other isothiocyanates have differing effects on cytochrome P450 activities depending on the protocol employed, the dose, and the time after dosing (Guo et al., 1993; Smith et al., 1990, 1993). In general, strong inhibitory effects are observed on pulmonary NNK metabolic activation, but the inhibition does not correlate with the effects of the isothiocyanates on specific cytochrome P450 enzymes known to be involved in NNK activation. These results suggest that there are unknown

cytochrome P450 enzymes in the mouse lung that metabolically activate NNK and are inhibited by isothiocyanates. Studies on the mechanisms by which PEITC inhibits BOP-induced hamster lung tumorigenesis conclude that PEITC exerts its chemopreventive activity by decreasing cell turnover and DNA methylation in the target organs, and by influencing hepatic cytochrome P450 enzymes (Nishikawa et al., 1997).

PEITC is a potent inhibitor of rat esophageal tumorigenesis induced by NBMA (Table 2). A comparative study demonstrates that PPITC is even more potent while BITC and PBITC have little effect on tumorigenesis (Wilkinson et al., 1995). PHITC enhances tumorigenesis in the same model (Stoner et al., 1995). Mechanistic studies clearly show that PEITC inhibits the metabolic activation of NBMA in the rat esophagus, probably through inhibition of a cytochrome P450 enzyme (Morse et al., 1997). Concomitant with this inhibition, one observes inhibition of O<sup>6</sup>-methylguanine formation in rat esophageal DNA (Wilkinson et al., 1995). The inhibitory effects: PPITC > PEITC > PBITC > BITC on tumorigenicity correlate with their inhibitory effects on O<sup>6</sup>-methylguanine formation (Wilkinson et al., 1995). In contrast, effects on carcinogen activation could not explain the enhancing effect of PHITC on rat esophageal tumorigenesis (Morse et al., 1997). Inhibition of NNN (*N*'-nitrosornornicotine) tumorigenicity in the rat esophagus also appears to be due to inhibition of its metabolic activation (Stoner et al., 1998).

Studies in humans who consumed watercress, a rich source of PEITC, support the results obtained in laboratory animals. A single oral dose of acetaminophen was given 10 hours after ingestion of watercress homogenates by a group of human volunteers. Watercress caused a decrease in the levels of oxidative metabolites of acetaminophen, probably due to inhibition of oxidative metabolism by P450 2E1 (Chen et al., 1996; Li et al., 1997). Consumption of watercress by smokers altered the profile of NNK metabolism, based on measurements of urinary metabolites (Hecht et al., 1995; Carmella et al., 1997). The results indicated that watercress consumption inhibited oxidative metabolism of NNK by inhibition of P450 1A2 (Hecht et al., 1995; Carmella et al., 1997; Smith et al., 1996). These results were consistent with observations in rats in which PEITC blocked NNK-induced lung tumorigenesis. Watercress consumption had no effect on P450 2D6 activity (Caporaso et al., 1994).

While these studies clearly show that inhibition of cytochrome P450 enzymes involved in the metabolic activation of carcinogens is a major mechanism by which isothiocyanates inhibit tumorigenicity, a series of recent investigations demonstrate another potential avenue of inhibition. PEITC is a strong inducer of c-Jun *N*-terminal kinase 1 (JNK1); this may be involved in the induction of Phase 2 enzymes (Yu et al., 1996). The sustained induction of JNK was associated with apoptosis induction in various cell types (Chen et al., 1998). Induction of apoptosis by isothiocyanates may proceed through a

caspase-3-dependent mechanism (Yu et al., 1998). PEITC blocks tumor promoter-induced cell transformation in mouse epidermal JB6 cells, and this inhibitory activity on cell transformation is correlated with induction of apoptosis. Moreover, apoptosis induction by PEITC occurs through a *p53*-dependent pathway (Huang et al., 1998). These events may be involved in chemoprevention by isothiocyanates and suggest that these compounds may have beneficial properties beyond their favorable modification of carcinogen metabolism.

## INDOLE-3-CARBINOL

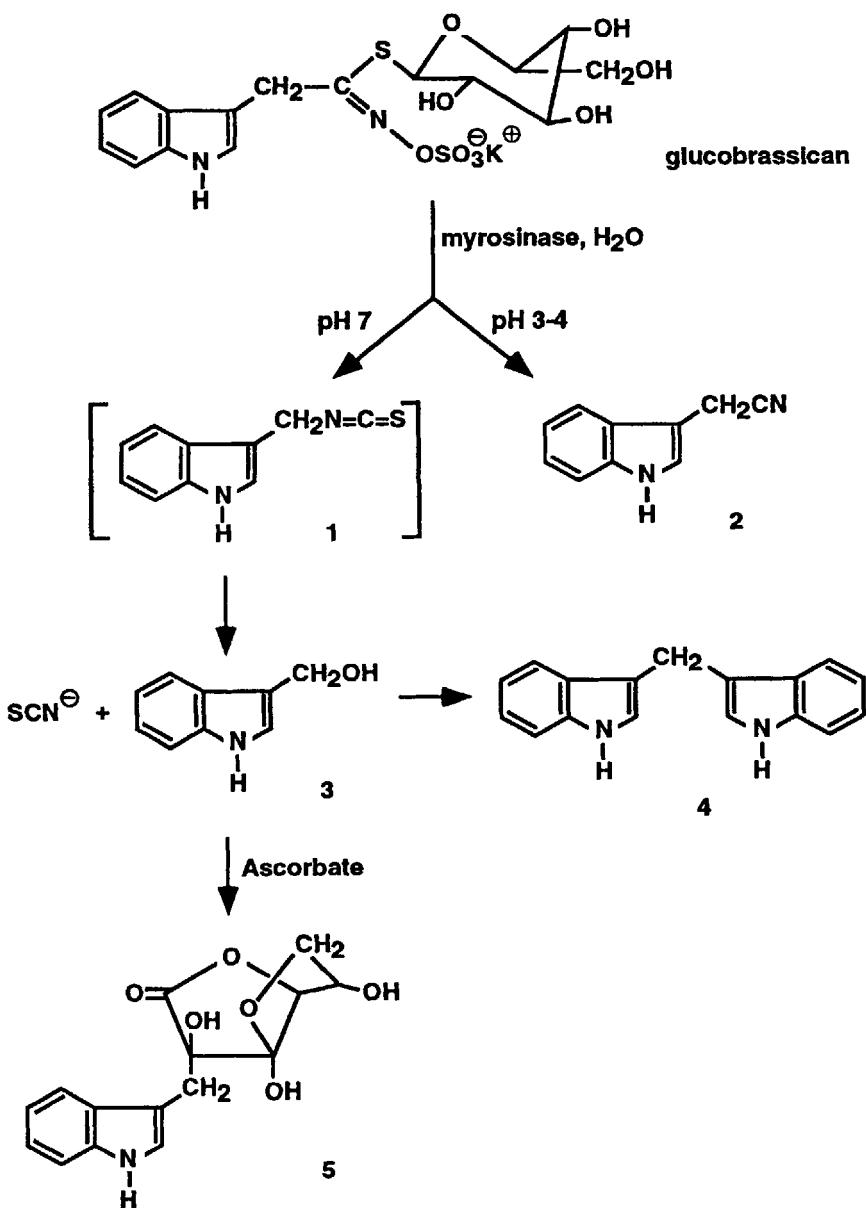
### OCCURRENCE AND FORMATION

Glucobrassicin, the glucosinolate precursor to indole-3-carbinol, is found in substantial quantities in a number of cruciferous vegetables. Typical levels in vegetables such as cabbage, cauliflower, brussels sprouts, and turnips range from 0.1 to 3.2 mmol/kg fresh plant weight (McDanell et al., 1988). In the United Kingdom, mean daily intake of glucobrassicin from cooked vegetables was estimated as 1.5 to 3.1 mg/person (Sones et al., 1984).

Myrosinase-catalyzed hydrolysis of glucobrassicin produces indole-3-carbinol and other products, as illustrated in Figure 2 (McDanell et al., 1988). At pH 7, the expected initial product is the corresponding isothiocyanate I but this has never been isolated or synthesized (McDanell et al., 1988). The isothiocyanate spontaneously hydrolyzes producing indole-3-carbinol (3). The latter self-condenses with the elimination of formaldehyde, producing 3,3'-diindolylmethane (4). When ascorbic acid is present, it reacts with indole-3-carbinol yielding ascorbigen (5). If the hydrolysis of glucobrassicin occurs at pH 3–4, indole-3-acetonitrile (2) is produced (McDanell et al., 1988).

### EFFECTS OF INDOLE-3-CARBINOL ON CARCINOGENESIS

Representative studies on the effects of indole-3-carbinol on carcinogenesis in animal models are summarized in Table 3. In most cases, indole-3-carbinol demonstrates inhibitory activity when given before or before and during carcinogen treatment. This is consistently observed in rat mammary tumor models using either directly or indirectly acting carcinogens, or in spontaneous tumor models (Wattenberg and Loub, 1978; Grubbs et al., 1995; Bradlow et al., 1991; Malloy et al., 1997). Other targets of inhibition are rat endometrium, mouse forestomach, rat liver, rat tongue, mouse lung, and trout liver (Wattenberg and Loub, 1978; Kojima et al., 1994; Tanaka et al., 1992; Tanaka et al., 1990; Kim et al., 1994; Pence et al., 1986; Nixon et al., 1984; Dashwood et



**Figure 2** Hydrolysis of glucobrassican to indole-3-carbinol.

TABLE 3. Modification of carcinogenesis by indole-3-carbinol.

Carcinogen <sup>a</sup>	Species and Target Organ	Effect	Reference
<b>A. Initiation Stage or Throughout</b>			
DMBA	Rat mammary	Inhibition	Wattenberg and Loub, 1978
MNU	Rat mammary	Inhibition	Grubbs et al., 1995
None	Mouse mammary	Inhibition	Grubbs et al., 1995
None	Rat endometrial	Inhibition	Bradlow et al., 1991
BaP	Mouse forestomach	Inhibition	Malloy et al., 1997
4-NQO	Rat tongue	Inhibition	Kojima et al., 1994
NDEA	Rat liver	Inhibition	Wattenberg and Loub, 1978
DMH	Rat colon	Inhibition	Tanaka et al., 1992
AFB <sub>1</sub>	Trout liver	Inhibition	Tanaka et al., 1990
NNK	Mouse lung	Inhibition	Kim et al., 1994
<b>B. Post-Initiation Stage</b>			
4-NQO	Rat tongue	Inhibition	Pence et al., 1986
NDEA + MNU + DHPN	Rat liver, thyroid	Enhancement	Nixon et al., 1984
NDEA	Rat liver	Enhancement	Dashwood et al., 1989
NDEA	Mouse liver	Inhibition	Morse et al., 1990a
AFB <sub>1</sub>	Trout liver	Enhancement	El-Bayoumy et al., 1996

<sup>a</sup> Abbreviations: see Table 2; additional abbreviations: MNU, *N*-methyl-*N*-nitrosourea; 4-NQO, 4-nitroquinoline 1-oxide; DMH, 1,2-dimethylhydrazine; AFB<sub>1</sub>, aflatoxin-B<sub>1</sub>; DHPN, dihydroxy-di-*N*-propylnitrosamine.

al., 1989; Morse et al., 1990a). Inhibition of carcinogenesis by a variety of different carcinogens including PAH, nitrosamines, nitro compounds, and aflatoxin B<sub>1</sub> is observed. An exception is DMH-(dimethylhydrazine) induced rat colon tumorigenesis, which was enhanced.

In contrast, there is strong evidence that indole-3-carbinol is a tumor promoter when given in the post-initiation stage, e.g., after carcinogen administration. This was first observed in the trout liver model and has been confirmed in rat liver and thyroid (Kim et al., 1994; Kim et al., 1997; Bailey et al., 1987; Dashwood et al., 1991). Other studies, however, demonstrate inhibition of mouse liver and rat tongue carcinogenesis by indole-3-carbinol given in the post-initiation stage (Tanaka et al., 1992; Oganesian et al., 1997).

Some unpublished studies support the results discussed above, and the U.S. National Cancer Institute is pursuing the clinical development of indole-3-carbinol. The target organ of highest clinical priority is the breast (National Cancer Institute, 1996a).

## MECHANISMS OF CHEMOPREVENTION BY INDOLE-3-CARBINOL

Mechanistic aspects of chemoprevention by indole-3-carbinol have been reviewed (McDanell et al., 1988; National Cancer Institute, 1996a; Bradfield and Bjeldanes, 1991; Williams et al., 1998). The protective effects of indole-3-carbinol against carcinogenesis result partly from its ability to modify enzymes involved in carcinogen metabolic activation and detoxification. In rats, indole-3-carbinol induces cytochromes P450 1A1, 1A2, 2B1, and 3A as well as Phase 2 enzymes, such as UDP-glucuronosyl transferase, epoxide hydrolase, and glutathione-S-transferases (Grubbs et al., 1995; Schertzer and Sainsbury, 1991a; Wortelboer et al., 1992; Stresser et al., 1994). It also induces P450s and Phase 2 enzymes in mice (Schertzer and Sainsbury, 1991b; Baldwin and Leblanc, 1992). Treatment with indole-3-carbinol reduces carcinogen-DNA adducts indicating that the overall modification of enzyme activities favors detoxification (Dashwood et al., 1989). Some of these effects are due not to the parent compound but rather to condensation products formed upon contact with gastric acid. Multiple products of this type are observed *in vivo* (Stresser et al., 1995). The acid condensation products are planar compounds that, unlike indole-3-carbinol itself, are agonists of the Ah receptor, resulting in the induction of P450 1A enzymes (Bradfield and Bjeldanes, 1991). 3,3'-Diindolylmethane, one of the acid condensation products, is also an inhibitor of rat and human cytochrome P450 1A1, human P450 1A2, and rat P450 2B1 (Stresser et al., 1995). The condensation products were more effective than indole-3-carbinol as inhibitors of aflatoxin-B<sub>1</sub> DNA binding and hepatocarcinogenesis in the trout, using an embryo microinjection model, indicating that their formation in the stomach is important in the expression of the biological activities of indole-3-carbinol (Dashwood et al., 1994).

While modification of carcinogen metabolic activation/detoxification ratios appears to be one mechanism of chemoprevention by indole-3-carbinol, changes in carcinogen distribution can also occur. In mice, indole-3-carbinol protects against NNK-induced pulmonary carcinogenesis by increasing the hepatic clearance of NNK and thereby decreasing bioavailability in the lung (Morse et al., 1990a). In these mice, urinary levels of two NNK metabolites—NNAL and NNAL-Gluc—decreased with a corresponding increase in levels of metabolites resulting from  $\alpha$ -hydroxylation (Morse et al., 1990a). In smokers treated with indole-3-carbinol, decreased levels of NNAL and NNAL-Gluc in urine were also observed, indicating that indole-3-carbinol has similar effects on hepatic NNK metabolism in humans and mice (Taioli et al., 1997). Enhanced metabolism of NNK in humans probably results from induction of P450 1A2 by indole-3-carbinol.

One of the indole-3-carbinol condensation products, indolo[3,2-*b*]carbazole, decreases estrogen receptor levels in cultured breast cancer cells (National Cancer Institute, 1996a; Liu et al., 1994). It is also a weak estrogen, but its action is mainly antiestrogenic in human breast cancer cells. Indole-3-carbinol also affects the metabolism of estradiol by increasing the ratio of 2-hydroxylation to 16- $\alpha$ -hydroxylation. 2-Hydroxylation is catalyzed by P450 1A2 and 16- $\alpha$ -hydroxylation by P450 3A4 (Yamazaki et al., 1998). Therefore, these results are consistent with induction of P450 1A2 by indole-3-carbinol. This ratio change is associated with inhibition of mammary and endometrial tumor development in rodents (Bradlow et al., 1985; Michnovicz and Bradlow, 1990). Indole-3-carbinol also enhances estradiol 2-hydroxylation in humans and has been proposed as a chemopreventive agent for breast cancer (National Cancer Institute, 1996a; Michnovicz and Bradlow, 1990; Bradlow et al., 1994; Michnovicz et al., 1997).

## THIOLS OF ALLIUM PLANTS

### OCCURRENCE AND FORMATION

Plants of the genus *Allium*, particularly garlic and onion, have been thoroughly investigated with respect to the occurrence of sulfur-containing compounds, which are responsible for their characteristic odors. This area has been reviewed (Fenwick and Hanley, 1985a–c; Block, 1992, 1996). When these plants are crushed, alliinases, which are C-S lyase enzymes, act on *S*-alkyl cysteine *S*-oxides to produce a wide variety of sulfur-containing compounds. Diallyl sulfide and related thiols have received the most attention with respect to chemoprevention.

### EFFECTS OF ALLIUM THIOLS ON CARCINOGENESIS

A number of studies demonstrate that onion and garlic oils inhibit tumorigen-

esis (Belman, 1983; Sadhana et al., 1988; Nishino et al., 1989; Perchellet et al., 1990; Liu et al., 1992; El-Mofty et al., 1994). This has spurred interest in chemoprevention by their constituents. Representative studies are summarized in Table 4. Diallyl sulfide is an effective inhibitor of tumorigenesis by a variety of carcinogen types including hydrazines, nitrosamines, aromatic amines, vinyl carbamate, PAH, and others. A large number of different tissues are protected including mouse colon, lung, skin, and forestomach and rat esophagus, lung, and thyroid. In general, diallyl sulfide inhibits tumorigenesis when administered prior to, or concurrently with, the carcinogen. For example, when given prior to NBMA, it is a potent inhibitor of esophageal tumorigenesis in the rat but has no effect when given after carcinogen administration (Wargovich et al., 1988, 1992). Both enhancement and inhibition have been observed in other studies in which diallyl sulfide was administered after the carcinogen (Jang et al., 1991; Takahashi et al., 1992; Takada et al., 1994).

Diallyl disulfide is also an effective inhibitor of tumorigenesis in mouse and rat models. However, diallyl trisulfide shows marginal effects or enhancement. Mixed results have been obtained with allyl methyl trisulfide, while allyl methyl disulfide and allyl methyl sulfide both are inhibitory in experiments reported to date. Saturated analogues are generally less effective as chemopreventive agents than the corresponding allyl compounds.

## MECHANISMS OF CHEMOPREVENTION BY ALLIUM THIOLS

Modification of carcinogen metabolism is the major mechanism by which diallyl sulfide and other *Allium* thiols protect against tumorigenesis. These compounds affect both Phase 1 and Phase 2 enzymes. Mechanistic studies on diallyl sulfide demonstrate that it is converted to diallyl sulfoxide and diallyl sulfone metabolically. Diallyl sulfide and diallyl sulfone are strong competitive inhibitors of cytochrome P450 2E1, which is involved in the metabolic activation of DMH, NDEA, and VC, three carcinogens that are inhibited by diallyl sulfide (Surh et al., 1995; Hong et al., 1994). Consistent with this, diallyl sulfide and diallyl sulfone are effective inhibitors of carbon tetrachloride, *N*-nitrosodimethyl-amine, and acetaminophen-induced hepatotoxicity (Hong et al., 1994). Diallyl sulfide probably inhibits P450s involved in the metabolic activation of NBMA and NNK as well, although these enzymes have not been fully characterized.

Inhibition of P450s is probably not the major mechanism by which diallyl sulfide and related compounds inhibit PAH tumorigenesis (Sparnins et al., 1988; Srivastava et al., 1997). Comparative studies demonstrate that induction of glutathione-*S*-transferase activity is the major protective mechanism operating in the mouse forestomach. Glutathione-*S*-transferases are involved in the detoxification of the diol epoxide ultimate carcinogens of PAH, such as BaP. A correlation has been observed between induction of glutathione-*S*-

TABLE 4. Modification of carcinogenesis by *allium* thiols.

Thiol	Carcinogen <sup>a</sup>	Species and Target	Effect	Reference
		Organ		
Diallyl sulfide	DMH	Mouse colon	Inhibition	Wargovich, 1987
	DMH	Rat liver	Inhibition	Hayes et al., 1987
	BaP	Mouse lung and forestomach	Inhibition	Sparnins et al., 1988
	NBMA	Rat esophagus	Inhibition	Wargovich et al., 1988
	NDEA	Mouse forestomach and lung	Inhibition or no-effect	Wattenberg et al., 1989
	NDEA, MNU, DBN	Rat, lung, thyroid	Inhibition	Jang et al., 1991
	NNK	Mouse lung	Inhibition	Hong et al., 1992
	NBMA	Rat esophagus	Inhibition or no effect	Wargovich et al., 1992
	DMBA	Hamster cheek pouch	Inhibition	Nagabhushan et al., 1992
	NDEA or NDEA, MNU, DMH, BBN, DHPN	Rat liver	Enhancement	Takahashi et al., 1992
	DMBA	Mouse skin	Inhibition	Dwivedi et al., 1992
	AA	Rat forestomach	Inhibition	Hadjilov et al., 1993
	IQ	Rat liver	Inhibition	Tsuda et al., 1994
	VC	Mouse skin	Inhibition	Surh et al., 1995
	NDEA	Rat liver	Enhancement	Takada et al., 1994
	NDEA	Mouse liver	Inhibition	Pereira, 1995

TABLE 4. (*continued*).

Diallyl disulfide	NDEA DMBA NDEA, MNU, DMH, BBN, DHPN	Mouse lung and fore- stomach Mouse skin Rat kidney and colon	Inhibition Inhibition Inhibition	Wattenberg et al., 1989 Dwivedi et al., 1992 Takahashi et al., 1992
Diallyl trisulfide	MNU BaP	Rat mammary Mouse lung and forestomach	Inhibition Inhibition (forestomach)	Schaffer et al., 1996 Sparnins et al., 1988
Allyl methyl trisulfide	NDEA BaP	Rat liver Mouse lung and forestomach	No effect (lung) Enhancement Inhibition (forestomach)	Takada et al., 1994
			No effect (lung)	Sparnins et al., 1986, 1988
Allyl methyl disulfide	NDEA BaP	Rat liver Mouse lung and forestomach	Enhancement Inhibition	Takada et al., 1994 Sparnins et al., 1988
	NDEA	Mouse lung and forestomach	Inhibition	Wattenberg et al., 1989
Allyl methyl sulfide	NDEA	Rat liver	Inhibition	Takada et al., 1994
Allyl mercaptan	NDEA	Mouse lung and forestomach	Inhibition	Wattenberg et al., 1989
Dipropyl trisulfide	BaP	Mouse lung and forestomach	Inhibition (forestomach)	Sparnins et al., 1988
Dipropyl disulfide	NDEA	Mouse lung and forestomach	Inhibition (forestomach)	Wattenberg et al., 1989
Dipropyl sulfide	DMBA BaP	Mouse skin Mouse lung and forestomach	No effect No effect	Belman et al., 1989 Sparnins et al., 1988

TABLE 4. (*continued*).

Thiol	Carcinogen <sup>a</sup>	Species and Target Organ	Effect	Reference
Propyl methyl trisulfide	NDEA	Rat liver	Enhancement	Takada et al., 1994
	BaP	Mouse lung and forestomach	No effect	Sparnins et al., 1988
Propyl methyl disulfide	BaP	Mouse lung and forestomach	No effect	Sparnins et al., 1988
Di (1-propenyl) sulfide	NDEA	Rat liver	Inhibition	Takada et al., 1994
	DMBA	Mouse skin	Inhibition	Belman et al., 1989
Ajoene	DMBA	Mouse skin	Inhibition	Belman et al., 1989
Propylene sulfide	NDEA	Rat liver	Inhibition	Takada et al., 1994

<sup>a</sup> Abbreviations: see Tables 2 and 3; additional abbreviations: DBN, dibutylnitrosamine; AA, aristolochic acid; IQ, 2-amino-3-methylimidazo [4,5-*f*]quinoline; VC, vinyl carbamate.

transferase activity and chemopreventive activity of a number of allyl thiols in the mouse forestomach, but not the lung (Sparnins et al., 1988). The allyl group is necessary for induction, which also parallels chemopreventive activity (Sparnins et al., 1988). In contrast, there is little effect of diallyl sulfide and related compounds on ethoxyresorufin O-deethylase or epoxide hydrolase activity (Srivastava et al., 1997).

Less is known about the effects of onion and garlic components on the post-initiation phase of carcinogenesis. One study shows that some of these compounds are effective inhibitors of soybean lipoxygenase activity. The strongest inhibitor, di(1-propenyl)sulfide, inhibited both lipoxygenase activity and tumor promotion in mouse skin while the corresponding saturated compound, di(*n*-propyl)disulfide, inhibited neither (Belman et al., 1989). Several sulfides that enhanced hepatocarcinogenesis by NDEA when given after carcinogen treatment also enhanced ornithine decarboxylase activity, but did not affect levels of 8-oxodeoxyguanosine or lipid peroxidation. These results suggest that the promoting effect of the sulfides could be caused by increased cell proliferation and polyamine biosynthesis (Takada et al., 1994).

## CONCLUSIONS

The results described here clearly demonstrate that Cruciferae and *Allium* plants as well as their constituents can inhibit carcinogenesis in a variety of animal models. The strongest evidence emanates from studies on the individual constituents, because variables can be more readily controlled. Isothiocyanates derived from naturally occurring glucosinolates are generally potent inhibitors of chemical carcinogenesis, particularly when administered prior to or concurrently with the carcinogen. The results suggest that isothiocyanates are stronger inhibitors of nitrosamine and PAH tumorigenesis than indole-3-carbinol or *Allium* thiols, but limited direct comparative data are available. For example, the lowest total gavage dose of PEITC required to significantly inhibit NNK-induced mouse lung tumorigenesis is 5 µmol, while the corresponding figures for indole-3-carbinol and diallyl sulfide are 100 and 105 µmol, respectively (Hecht, 1998b). BITC also appears to be a stronger inhibitor of mouse lung tumorigenesis than diallyl sulfide (Wattenberg, 1977; Lin et al., 1993; Sparnins et al., 1988). There are few examples of enhancement of carcinogenesis in studies with naturally occurring isothiocyanates or *Allium* thiols. In contrast, there can be little doubt that indole-3-carbinol has tumor-promoting activity. On balance, however, the available data are consistent with the hypothesis that specific chemopreventive agents in vegetables are at least partially responsible for the protective effects of vegetables against cancer that are seen in epidemiologic studies.

There are many complexities in attempting to evaluate the potential anticarcinogenic effects of vegetables. First, levels of specific chemopreventive agents

vary widely depending on the particular species and cultivar. Cooking and eating conditions will also affect the uptake of specific agents. There are interindividual differences in metabolism of the chemopreventive agents. For example, a recent study suggests that people who are deficient in GSTM1 and who consume broccoli are protected against colon cancer because of less efficient metabolism of chemopreventive isothiocyanates (Lin et al., 1998; Ketterer, 1998). Another study demonstrates higher P450 1A2 activity in individuals who consume cruciferous vegetables and are GSTM1 null, presumably due to induction by isothiocyanates or related compounds (Probst-Hensch et al., 1998). Human exposure to carcinogens is also complex as is the metabolism of each carcinogen, where there are large inter-individual differences in multiple pathways of activation and detoxification. Measurement of human uptake and metabolism of chemopreventive agents in vegetables is necessary for evaluating the potential anti-carcinogenic effects of vegetables. Few studies of this type have been carried out for the agents considered here, but new methods for assessing isothiocyanate uptake are becoming available (Chung et al., 1992; Zhang et al., 1996; Chung et al., 1998). It will be important to incorporate these into epidemiologic studies that also employ biomarkers of carcinogen metabolism.

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# **Clinical Trial Design for Evaluating Phytochemical Bioactivity**

**KEVIN C. MAKI**

## **INTRODUCTION**

**T**HE randomized, controlled trial is the established “gold-standard” for evaluation of interventions intended to influence human health. Recent technological and regulatory developments have resulted in the identification and commercial development of an ever-increasing number of bioactive phytochemicals with the potential for application in the prevention and/or treatment of disease. Evidence from animal models, *in vitro* studies, biochemical investigations, and epidemiologic investigations provide important data that may be useful for evaluating the safety and efficacy of phytochemical products. Nevertheless, regulatory bodies such as the United States Food and Drug Administration and Federal Trade Commission stipulate that substantiating claims of efficacy and/or safety for phytochemical products requires direct evidence from randomized, controlled studies involving human subjects, i.e., clinical trials. The objective of this chapter is to review fundamental concepts in clinical trial methodology and provide practical suggestions for application of these principles to clinical trials intended to assess the bioactivity of products containing phytochemical compounds.

## **CLINICAL TRIAL: A DEFINITION**

The term “clinical trial” may be applied to any form of planned experiment that involves a sample of human subjects and is designed to elucidate the

most appropriate intervention for the prevention or treatment of a medical condition (Table 1, adapted).

## FUNDAMENTAL PRINCIPLES

Systematic application of clinical trials to research and development functions was not widespread until the 1950s. Today, clinical trials are widely employed for evaluating the safety and efficacy of pharmaceutical products, medical devices, surgical procedures, food additives, medical foods, and dietary supplements. Specific guidelines for Good Clinical Practice and recommendations for clinical trial design and conduct have been issued by the Food and Drug Administration, as well as international groups such as the World Health Organization and the International Committee on Harmonization. Several features may be considered central to the design and conduct of any clinical trial, regardless of the intervention under study. These criteria will be explored in some detail throughout this chapter, including the following:

- *a priori* development of a focused, explicit research question
- identification of pre-defined, objective outcome measures
- use of appropriate controls
- random assignment to treatments or treatment sequences
- blinding of the investigators and subjects whenever feasible
- inclusion of a large enough subject sample to have adequate statistical power to detect clinically important differences between treatments or treatment sequences

TABLE 1. Main features of clinical trial protocol

- |   |
|---|
| <ul style="list-style-type: none"> <li>• background and general aims</li> <li>• specific objectives</li> <li>• subject selection criteria</li> <li>• treatment schedules</li> <li>• methods of patient evaluation</li> <li>• trial design</li> <li>• registration and randomization of subjects</li> <li>• informed consent form and institutional review board review</li> <li>• number of subjects and justification thereof</li> <li>• plan for monitoring</li> <li>• procedure for handling protocol deviations</li> <li>• plans for statistical analysis</li> <li>• administrative responsibilities</li> <li>• data collection forms and plan for data handling</li> </ul> |
|---|

## REGULATORY ISSUES

The motivation for undertaking a clinical trial to investigate the biological activity of a phytochemical is most often to collect data to satisfy regulatory requirements and/or support marketing claims. From a regulatory standpoint, the manufacturer or marketer of a new product will be held accountable for collection of the necessary clinical trial data to demonstrate that the product is safe and effective for its intended use. The specific clinical trial data required to satisfy regulatory requirements will depend upon how the product is classified and its method of administration. A product containing one or more bioactive phytochemicals may be considered a drug, cosmetic, dietary supplement, food, medical food, infant formula, or food additive. Review of the regulatory process is beyond the scope of this chapter. The interested reader is referred to an excellent book entitled *A Practical Guide to Food and Drug Law and Regulation* for additional information (Pina and Pines, 1998). In addition, the Federal Trade Commission has recently issued *Dietary Supplements: An Advertising Guide for Industry* (Federal Trade Commission Bureau of Consumer Protection, 1998), which outlines the requirements for substantiation of advertising claims for dietary supplement products.

## SETTING

One of the first decisions faced by an investigator or clinical trial sponsor is to determine the setting in which the trial will be conducted. Clinical trials may be performed at an outpatient clinic, metabolic ward, or hospital. Metabolic wards are appropriate for trials that require a high degree of control over variables such as dietary intake and physical activity or when participants must be monitored intensively, as is the case for first-time use of new pharmaceutical products in human subjects. A hospital setting may be required for evaluation of therapies, procedures, or devices when the participants are acutely ill or undergoing medical procedures. However, a majority of clinical trials are conducted at outpatient clinics.

Outpatient clinic and metabolic ward studies may be undertaken by investigators in a university or academic medical center. Alternately, the investigators and study staff may be employed at an independent clinical research center that is not affiliated with a university. These centers are often associated with physician practices, but may be devoted entirely to running clinical trials. Independent research centers are used extensively for pharmaceutical development studies.

The most appropriate setting for a particular trial is determined by a number of factors, including expertise, experience, prestige, timeline, and cost. Academic investigators may offer prestige and a high level of expertise. Compared

to academic settings, independent research centers often have greater capacity to initiate a trial quickly and to rapidly recruit study participants. However, the cost of conducting a clinical trial at an independent research center is often, but not always, greater than that of an academic center.

For large, multicenter trials, a contract research organization (CRO) may be employed to coordinate the administration of the study. Typical CRO functions include protocol development, site identification, study monitoring, data management, statistical analysis, and final report generation.

## **THE CLINICAL TRIAL PROTOCOL**

The clinical trial protocol is a document that outlines the research question, specific objectives of the study, the trial design, number of subjects, and inclusion/exclusion criteria, as well as providing the details of how the study will be conducted. Large or complex trials may also have an operational manual that provides further details and procedures for the trial. The main features of a clinical trial protocol are summarized in Table 1. The trial protocol may be prepared by the study sponsor, which is often the case for pharmaceutical development studies, or by the investigator(s).

Literally hundreds of decisions are required in order to develop a research question into a complete clinical trial protocol. These fall mainly into five broad categories:

- outcome variables
- study design
- inclusion/exclusion criteria
- blinding and controls
- statistical power and sample size

## **OUTCOME VARIABLES**

Objective outcome variables should be clearly specified in the study protocol. While it is not uncommon to measure a variety of indicators of treatment response, interpretation of the study results is aided by clearly identifying the variable(s) of primary interest during the design phase. Whenever possible, one or two measures of response should be identified as primary outcomes. Other response indicators may be considered secondary or supportive. For example, a study assessing the influence of a phytochemical product on the serum lipid profile may specify the percent change from baseline in low-density lipoprotein cholesterol concentration as the primary outcome variable. Percent changes in high-density lipoprotein cholesterol and triglyceride levels may be secondary outcomes.

Clinical trials are expensive and time consuming to conduct. For these reasons, investigators are often interested in including extra measurements that are not central to the primary research question(s). Such ancillary studies often provide clinically and scientifically important information. However, care should be taken so that collection of the data necessary for ancillary studies does not jeopardize the main objective of the trial by placing excessive burdens on the study subjects or staff.

For most clinical trials, it is possible to conduct many statistical evaluations after completion of the study, some of which may not have been envisioned at the time the study was designed. Statistically significant results for subgroup analyses or analyses of research questions not specified in the study protocol (sometimes referred to as "data dredging") should be interpreted with caution and should always be subject to verification. The reason for this is that the probability of finding spuriously significant results increases with the number of statistical tests performed. If 100 hypothesis tests are completed, approximately five would be expected, by chance, to show statistical significance at the 5% level.

## STUDY DESIGN

Parallel studies include concurrent enrollment of subjects assigned to one or more active treatments and subjects assigned to one or more control conditions. Crossover trials allow each subject to act as his or her own control. For a crossover trial, a subject may undergo control treatment (e.g., placebo) and one or more active treatments. Whereas the treatment group is chosen by random assignment in a parallel study, the treatment sequence is assigned at random in a crossover trial.

Because within-subject responses generally show less variability than those between subjects, fewer participants are required for a given level of statistical power when a crossover design is employed. Despite this attractive feature, crossover trials have limited usefulness for evaluating efficacy. Crossover studies are most useful when limited to short treatment periods for relatively stable, chronic conditions such as hypercholesterolemia or hypertension. The risk of generating results that are not clearly interpretable is much higher with crossover designs compared with parallel studies.

Two assumptions inherent in evaluation of crossover trial results are that no period or carryover effects are present. The assumption of "no period effect" refers to the idea that all subjects' underlying condition and ability to respond remain unchanged between treatment periods. However, a subject's underlying condition may improve or deteriorate over time, rendering this assumption invalid. Carryover effects occur when the influence of one treatment extends into the next treatment period. If this occurs, responses may

vary according to the sequence in which the treatments are given. Thus, in a study with two treatments, active and placebo, responses may differ between those in the active-placebo sequence compared with subjects in the placebo-active sequence. One way to limit the possibility of carryover is to lengthen the washout period between treatments. However, doing so may also increase the possibility that a ‘‘period effect’’ will be observed and, by lengthening the duration of each participant’s commitment, may increase subject attrition.

In any trial, whether of parallel or crossover design, the length of treatment should be sufficient to produce clinically relevant results. For example, some antidepressant medications take two to three weeks to produce a measurable effect. Therefore, a trial with a treatment period of only one week would be unlikely to support the efficacy of the medication under study. However, compliance with the study regimen may deteriorate with time. Therefore, initial efficacy studies should not be of such a long duration that lack of compliance might be expected to confound the results. Once efficacy has been demonstrated in a relatively short trial, longer trials may be undertaken to determine the practicality of maintaining this effect over an extended period.

Prior to randomization, a lead-in (also known as a run-in) period may be employed to allow time for demonstration of a stable baseline for outcome variables and potential confounders (e.g., body weight). Often, subjects will be given a placebo during the lead-in period. Those who are shown to be poorly compliant during the placebo lead-in may be excluded from participation before being randomized. In addition, use of a placebo allows the investigators to identify subjects who are highly ‘‘suggestible’’ and prone to report adverse experiences.

## INCLUSION AND EXCLUSION CRITERIA

Specific inclusion and exclusion criteria, which define the characteristics of the subjects to be included in the trial, are outlined in the study protocol. A chief objective is to define a sample that will be representative of the group to whom the trial’s findings may be applied. Therefore, strict, objective criteria should be developed. If, for example, the intervention under investigation is intended to lower blood cholesterol concentration, the following inclusion criteria might be employed:

- (1) Apparently healthy men and women, 18 to 65 years of age
- (2) Fasting low-density lipoprotein (LDL) cholesterol concentration 130 to 190 mg/dL based on the average from measurements obtained at clinic visits one (week 6) and two (week 5)
- (3) Body mass index 21.0 to 32.0 kg/m<sup>2</sup>
- (4) People to whom the nature of the study has been fully explained and who are capable of providing informed, written consent to participate

These criteria illustrate several points. The age range is clearly defined and specifies that people of both genders are eligible to participate. The range of the baseline LDL cholesterol level is clearly defined. The parameters and ranges selected should have clinical relevance. In this case, the National Cholesterol Education Program Adult Treatment Panel (1993) report was used to define levels of LDL cholesterol considered undesirably high, but not so high that failure to institute drug therapy might put the participant at undue risk. Consensus statements or treatment recommendations from authoritative medical or scientific bodies should be used whenever possible to define inclusion criteria. Some examples include the National High Blood Pressure Education Program, the American Diabetes Association, the American Cancer Society, etc.

Specific ranges should be specified for variables that might be reasonably expected to influence the response to treatment. In the example above, eligible subjects must fall within a defined range for body mass index in order to exclude those subjects who are at the extremes of body weight relative to height. Other variables commonly specified include gender, age, and lifestyle habits (e.g., physical activity and diet). All participants must be fully informed of the study's objectives and procedures and be capable of understanding and voluntarily signing an informed consent document. Special procedures need to be implemented for studies involving children or those who are incapable of providing informed consent, such as a patient with a medical condition that might preclude understanding the potential risk and benefits of participation (e.g., comatose patients or those with severe schizophrenia, Alzheimer's disease, etc.).

Exclusion criteria are used to specify conditions or characteristics that would disqualify someone from participation. These are chosen to exclude from participation those people with factors that might interfere with the interpretation of the study results, represent undue risk, or reduce the probability that a subject will complete the trial. General categories include the following:

- medical conditions
- concomitant medications, therapies, or dietary supplement use
- planned or recent changes in the person's personal situation (e.g., planned relocation before the end of the treatment period, large weight changes, recent smoking cessation, pregnancy, etc.)
- extreme dietary or physical activity patterns
- alcohol or drug abuse

While it is desirable to define the exclusion criteria as objectively as possible, no list will ever be sufficient to anticipate every situation. Therefore, some latitude must be allowed for the judgment of the investigator.

The importance of carefully considering the inclusion and exclusion criteria

cannot be overstated. It may be useful to consider the following list of questions when evaluating the inclusion and exclusion criteria for a clinical trial:

- Is the group identified by these criteria representative of the target population to whom these results will be generalized in terms of demographic variables and baseline severity of the condition under study?
- Are the criteria so restrictive that enrolling the necessary number of subjects will exceed the available time and budget?
- Has the influence of all major variables with potential to influence the response been considered in development of these criteria?

## **BLINDING AND CONTROLS**

Whenever feasible, both the participants and investigators should be unaware of the treatment to which subjects have been assigned. For pharmaceutical studies, this is often accomplished through use of a matching placebo tablet or capsule. Producing matched placebos for phytochemical products may not always be possible. If the product under study is a food, the sensory qualities may not be possible to mask. In other cases, the study product may have side effects that make blinding difficult or impossible. For instance, trials investigating the blood lipid-altering effects of niacin were not always possible to blind because of the characteristic skin flushing associated with administration of high doses of niacin.

It is never possible to institute controls for all variables that potentially influence response to treatment. If the study sample is large enough, the distribution of factors with the potential to influence the treatment response has a low probability of showing imbalances among treatment arms. With smaller study samples, there is a greater risk that clinically important differences between treatment groups will occur. Stratified randomization or matching may be used to ensure balanced distribution of important factors between treatments. For some variables that may influence treatment response, it may not be possible or practical to institute strict controls, e.g., dietary habits, alcohol consumption, physical activity, etc. Measurement of potential confounders at baseline and one or more times during treatment will be useful for assessing the possible impact of these factors when the data are analyzed.

## **SPECIAL CONSIDERATIONS FOR INVESTIGATIONS OF PHYTOCHEMICALS IN FOODS**

Phytochemical products are often delivered in the form of a food. Several issues must be considered when designing trials to test functional foods. First,

whenever a food is added to the diet, its energy content must displace those from other foods or beverages. Care should be taken to ensure that addition of the study product does not disrupt the diet to the extent that unwanted physiological consequences occur. Unwanted consequences may include weight gain, changes in the distribution of macronutrients, or reductions in consumption of nutritional factors that might influence the study results. In one trial conducted at our center, a dietary fiber supplement was delivered in an apple juice vehicle. However, the high sugar and energy content of the apple juice disrupted the diet to the extent that body weight and blood lipid levels increased, complicating interpretation of the study results (Davidson et al., 1998).

Ideally, control foods would be prepared that are indistinguishable from the food containing the compound under study. However, this is not always possible. For example, our center conducted a trial assessing the influence of dietary fiber from oat products on blood lipids (Davidson et al., 1991). Because the active treatments (oatmeal and oat bran) have distinctive sensory qualities, it was not possible to create "placebo foods." Accordingly, a low fiber wheat cereal was used as a control in order to maintain a close match between the energy and nutrient composition of the food products under study.

## SAMPLE SIZE AND POWER

Few decisions cause more anguish than those relating to the number of subjects that should be included in a clinical trial. Studying too many subjects increases the cost unnecessarily. Including too few subjects can result in a non-significant result, even though the treatment may be effective. Estimation of the appropriate sample size involves several assumptions.

First, the effect (difference between treatments) that is anticipated for the primary outcome variable should be estimated from a pilot trial or previously published results. Detection of small effects requires large samples, whereas fewer subjects are needed to detect large effects. Generally, one should design the trial to have the statistical power to detect a smaller response than anticipated. Thus, if a 10% reduction in LDL cholesterol is anticipated for the active treatment, with no change in the placebo group, it may be prudent to design the trial to have adequate power to detect a difference of 7% in LDL cholesterol response between treatment arms. Moreover, the potential for a "placebo effect" should be factored into the anticipated treatment response. For outcomes with a subjective component, e.g., subject ratings of the frequency and intensity of symptoms, it is not uncommon to observe substantial improvement (30 to 60%) among subjects taking a placebo. Examples of conditions for which a placebo effect on outcomes might be expected include arthritis, angina pectoris, depression, premenstrual syndrome, hot flashes, claudication,

impotence, benign prostatic hyperplasia, and migraine headaches. Any effect of the treatment under study will need to be demonstrated above and beyond that of the influence of placebo.

The second issue that needs to be considered is the variability of the outcome. Variability in the treatment response occurs due to biological variation, presence of subgroups of non-responders or hyper-responders, and precision of the measurement tools employed. Greater variability in response is associated with the need for a larger sample to demonstrate statistical significance for a given magnitude of effect. Therefore, efforts to limit variability will help to maximize the probability of detecting a treatment effect and will reduce the number of subjects required.

The influence of biological variability may be minimized by averaging the values from multiple measurements at baseline and during treatment. For example, if the primary outcome variable for a trial is the percent reduction from baseline in LDL cholesterol, two or three measurements obtained on different days may be averaged for the baseline and end-of-treatment values. An additional means by which variability is minimized is to restrict the characteristics of the study sample. Trials are generally designed to include only those subjects who are most likely to respond to the treatment under investigation. A trial investigating the cholesterol-lowering influence of a phytochemical product might exclude subjects with low or normal cholesterol levels at baseline, who may be less likely to respond to treatment than those with hypercholesterolemia.

For some variables, several measurement tools may be available with which to assess response. As an example, body fat mass may be estimated by dual x-ray absorptiometry, hydrostatic weighing, bioelectrical impedance analysis, or skinfold assessment. These tests vary considerably in precision, as well as cost. Use of more precise tools reduces the number of subjects required for the trial. Therefore, the cost of obtaining the measurements with more precise and expensive tools must be balanced against the cost of recruiting additional subjects.

The specifics of determining sample size and power for clinical trials are beyond the scope of this chapter. The interested reader is referred to texts by Glantz (1997), Hulley and Cummings (1988), and Pocock (1983) for additional information.

## BUDGET

The main determinants of clinical trial costs are the number of subjects enrolled, the number and complexity of the clinic visits each participant will undergo, the cost of measurements, including laboratory analyses, (e.g., X-rays, medical procedures), and subject remuneration (e.g., reimbursement for

travel expenses and/or a stipend for participation). Additional costs include data management, statistical analysis, and medical writing (e.g., study protocol, final report, manuscript for publication).

## **SEEK EXPERT ADVICE**

Clinical trials are expensive and time consuming to conduct. The importance of seeking expert advice when designing a trial cannot be overstated. Experienced investigators are able to provide invaluable insight regarding inclusion and exclusion criteria, ease of recruitment, patient burden for specific procedures, strategies to enhance subject adherence, advantages and disadvantages of various measurement options, etc. In particular, a qualified statistician should be consulted regarding sample size and power calculations, after which a second opinion should be obtained.

## **PUBLICATIONS**

The investigator's responsibility for a clinical trial does not end when the last subject completes the study. Clinical trial findings cannot be applied to improve the health of men, women, and children until the results are communicated to those who are in a position to make use of the data, including scientists, industry personnel, clinicians, and the public. Presentation to scientific/medical bodies and publication in peer-reviewed journals are imperative.

## **SUMMARY**

The randomized, controlled trial remains the "gold standard" for evaluation of interventions designed to favorably influence human health, including phytochemical products. The design and implementation of a clinical trial involves hundreds of decisions. The trials most likely to achieve their stated objectives cost-effectively will be designed according to the fundamental principles outlined herein. Careful consideration of the research question, outcome measures, control conditions, randomization, blinding, inclusion/exclusion criteria, and statistical power are necessary components of the process. Collaboration and consultation with expert clinicians and scientists with extensive experience in clinical trial design and analysis will help maximize the probability of success.

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# **The Use of Fermentable Fibers to Manage the Gastrointestinal Ecosystem**

RANDAL K. BUDDINGTON

## **THE GASTROINTESTINAL TRACT IS AN ECOSYSTEM**

**T**HE combination of longer lifespans and lower concentrations of protective phytochemicals in the modern diet are correlated with an increased risk of cancer. Furthermore, epidemiologic studies have shown population-based differences for incidences of cancer that can be attributed to levels of dietary fiber (Dwyer, 1993). This has led to an increasing awareness of the interactions between diet and health (Adlercreutz, 1998; Kelly et al., 1994). It is now recognized that the bacterial assemblages present in the gastrointestinal tract (GIT) and the associated metabolic activities are important determinants for the risk of large bowel cancer (Gorbach and Goldin, 1990; Bartram et al., 1993; Moore and Moore, 1995). A common goal is to identify dietary inputs that can be used to effectively manage the GIT to encourage health and reduce the risk of disease.

The GIT can be considered as a small, but complex and dynamic, ecosystem (Bry et al., 1996). Although this concept is not new (Haenel, 1961), the interactions between dietary inputs and the host organism are not yet fully understood. The GIT shares several similarities with river ecosystems, and the application of ecological principles is appropriate for gaining an understanding of the interactions between the different components of the GIT and the influences of exogenous inputs (Buddington and Weiher, 1999). Rivers and the GIT are continua with unidirectional flow that extends from a source (lakes or reservoirs vs. the stomach) through a channel with changing structural, functional, and chemical characteristics. The transitions between adjacent

regions range from very gradual and almost imperceptible (e.g., jejunum and ileum) to abrupt and dramatic (e.g., stomach and duodenum).

The objective of this chapter is to describe how fermentable fibers can be used as a tool to manage the GIT ecosystem to promote health and reduce the risk of disease. Readers are first familiarized with the concept of the GIT as an ecosystem and the possible “tools” that can be used for management purposes. The use of fermentable fibers, with an emphasis on fructooligosaccharides (oligofructose), is then described as a tool for managing the GIT in mature individuals, during development, in senescence, and after a disturbance. The goal of this review is to provide a foundation of information for readers interested in learning more about how diet can be used to manage the GIT. Although the references are not exhaustive, they should assist readers in locating additional information.

## **COMPONENTS OF THE GIT ECOSYSTEM**

Ecosystems are considered to consist of structural and functional elements. Whereas the structural elements include the biotic and abiotic components, the functional elements involve the flow of materials and energy between compartments. Both are readily apparent in GIT and rivers.

The GIT and rivers have horizontal gradients that include several regions that represent separate habitats with distinct characteristics. Flow is considered to be unidirectional, but there can be localized regions or limited periods with reverse flow. Another gradient that is not as obvious, but is just as important, is the vertical gradient that extends from the mucosa up into the lumen, much like the vertical gradient in streams from the sediments into the water column.

## **THE ABIOTIC COMPONENTS**

The physical features of the GIT are critical determinants for the composition and distribution of organisms. The basic structure and functions of the GIT are set by genetic determinants that vary among species. Even within a species, there are differences between stages of development and individuals. Although the GIT is dynamic, there is a limit to the magnitude of change that can be induced by dietary inputs. Another important feature is the mucosal architecture and the degree of complexity. Similar to the shore of rivers (riparian zone), the mucosa of the GIT is the site of exchange, and it regulates the movement of materials between the organism and the external environment. The mucosa also serves as a refuge and site of attachment for organisms, much like the sediments of a river.

The viscosity of the digesta, the chemical composition (e.g., pH, types and concentrations of organic and inorganic constituents, oxygen content, redox

potential, water content), and rate of movement vary in the different regions of the GIT that influence densities of bacteria (Simon, 1998). These characteristics of the digesta reflect dietary inputs (amount and composition) and the secretory and absorptive functions of the GIT.

## BIOTIC COMPONENTS

Bacteria are considered as the dominant group of organisms, numbering more than the cells in the host's body. More than 400 species have been identified, and it is expected that many more will be isolated and identified with advances in techniques for culture and identification. Much like the densities of organisms vary along the length of a river, the densities of bacteria vary along the length of the GIT, ranging from less than 1000/ml in the stomach up to  $10^{9-12}/\text{ml}$  in the colon (Toskes, 1993).

The assemblages of bacteria also vary in the different GIT regions. For example, species tolerant of oxygen dominate in the stomach and proximal small intestine, whereas strict and facultative anaerobes comprise the majority of the bacteria in the colon. Even in a region, there are differences between bacteria present in the lumen versus those associated with the mucosa, much like organisms in the water column of a river differ from those associated with the sediments.

There are three principle factors that influence bacterial assemblages in the GIT. First, the anatomy and physiology of the host provide the basic environment, much like the geographical location of a river is critical for determining the resident species. The second is the amount and composition of the diet. Finally, there are the interactions among the bacteria themselves. Notable are the inhibitory influences of *Bifidobacteria* on proliferation of other bacterial groups, particularly several considered to be pathogenic (Gibson and Wang, 1994), with similar findings for the *Lactobacilli* (Juven et al., 1991).

Although at first sight the bacterial assemblages in the GIT of humans appear to be comparable, there are subtle differences between individuals. In a similar manner, streams in the same geographical region may share many abiotic features, but more often than not can be distinguished from each other by subtle to large differences in biotic components. These can result in dramatic differences in the functional elements of the transfer of energy and materials. When one considers the metabolic capacities of the bacteria, their dynamic nature, and the interactions with other host systems, the GIT bacteria can be considered as another "organ."

The species assemblages and metabolic characteristics of the GIT bacteria influence health directly and indirectly by influencing the activities of enzyme systems associated with the mucosa of the host (Kinouchi et al., 1993; Abrams, 1977). Exemplary is overgrowth of the small intestine, which is associated with changes in the densities and assemblages of the bacteria in the small

intestine, such that they are similar to those typical for the colon, and is often associated with dysfunction of small intestine functions. The GIT bacteria are also involved in host nutrition (Savage, 1986) and metabolic processing (activation/deactivation) of carcinogens present in the diet (Kautiainen et al., 1993). Furthermore, it is now recognized that the pathogenesis of Crohn's disease is related to GIT bacteria (Favier et al., 1997). Corresponding with these findings, any disturbances that perturb the normal GIT bacteria can have profound impacts on health. Therefore, interventions that improve the composition and metabolic activities of the GIT bacteria or accelerate their recovery during or after GIT diseases should provide health benefits.

## **GASTROINTESTINAL FUNCTIONS**

In addition to digestion, the GIT is involved in immunity and osmoregulation and is considered to be the largest endocrine organ in the body. These functions largely reside in the mucosa. As a consequence, there are complex interactions between the composition of the diet, the resident bacteria, and GIT functions (Macfarlane and Cummings, 1991; Simon and Gorbach, 1987). The most dramatic examples are those of food poisoning, with pathogens eliciting responses by the absorptive, secretory, immune, and endocrine functions of the GIT.

Certain bacterial groups are considered to provide benefits. This includes the purported abilities of lactic acid producing bacteria to enhance enteric defense mechanisms (De Simone et al., 1987; Gaskins et al., 1996; Perdigón et al., 1993), as well as systemic immunity (Schiffrin et al., 1997). Bacteria also influence enteric endocrine functions (Pen and Welling, 1983). Moreover, short-chain fatty acids (SCFA) and possibly other bacterial metabolites trigger the release of bioactive peptides (e.g., glucagon-like peptide 2) that stimulate growth and nutrient transport functions of the proximal small intestine (McBurney et al., 1998).

## **MANAGEMENT TOOLS**

Management of ecosystems, whether the GIT or a river, is ultimately directed at manipulating the composition and densities of the various resident organisms. The objective is to encourage the proliferation and growth of desirable species and inhibit or eliminate those considered to be detrimental. The benefits of successful management include greater ecosystem productivity, increased efficiency of utilization of inputs, improved "health," and resistance to invasion by less desirable species. Several approaches are used to manage the GIT ecosystem.

Antibiotics, like pesticides, can be used for selective removal of target species, and they provide benefits when added to animal diets (e.g., growth promotion and increased feed efficiency). However, there is growing concern about their use because of the development of resistant strains of bacteria and the potential impact on other ecosystems. Another detriment of some antibiotic therapies (oral and systemic) is the disruption of the normal bacterial assemblage (Nord, 1993). There are alternatives to antibiotics. Lectins, certain monosaccharides (e.g., mannose), and organic acids (mono-, di-, and tricarboxylic acids) can be used to selectively decrease the densities of some pathogenic bacteria (Russell and Diez-Gonzalez, 1998; Kelly, 1998).

A common strategy to manage rivers and other ecosystems is to simply add desirable species. The use of probiotics (adding viable bacteria) is analogous for managing the GIT. The benefits of probiotics are established and include stimulating immune functions (De Simone et al., 1987), altering the metabolic activities of the GIT bacteria (Ling et al., 1994), and effectively changing the GIT environment. Probiotics are often added to dairy products and may be of particular benefit to individuals suffering from diarrhea (Kimura et al., 1983) and to infants (Guerin-Danan et al., 1998). The drawback of probiotics is that the benefits are transient, lasting only for as long as the bacteria are ingested (Ling et al., 1994).

The numerous growth factors known to influence the GIT (Odle et al., 1996) may prove to be useful management tools during development and for recovery from GIT disease. By accelerating growth and functional maturation, growth factors effectively alter the physical and chemical features of the GIT ecosystem (Burrin et al., 1996). Although not established, it can be predicted that by doing so growth factors may hasten the development of the normal bacterial assemblages.

The presence of luminal nutrients is critical for normal GIT structure and functions. Similar to rivers without water, the lack of nutrients in the GIT causes marked changes in the structure, functions, and populations of resident bacteria. Exemplary are the disturbances caused by total parenteral nutrition (TPN) when the lack of luminal nutrients causes the mucosal barrier to be compromised and increases the risk of bacterial translocation and septicemia (Zaloga et al., 1993). Nutrients must also be present in proper balance to maintain mucosal structure and functions, trigger digestive secretions, and sustain the normal GIT bacteria (Gorbach and Goldin, 1992). Bacteriologic analysis of stool samples suggests that large-scale changes in dietary inputs are needed to elicit marked changes in the bacterial assemblages (Toskes, 1993). However, smaller scale shifts in nutrient inputs can cause detectable changes in the composition and metabolic characteristics of the GIT bacteria (Buddington and Sunvold, 1998), with the influences more profound in the small intestine (Buddington, 1998). Corresponding with this, adding micronutrients to or deleting them (e.g., Cu and Fe, respectively) from the diet of

infants can influence development of the GIT bacteria (reviewed by Buddington, 1998). Another dietary approach is the addition of exogenous enzymes to elicit changes in the physical and chemical characteristics of the luminal contents (Simon, 1998), which in turn influences the resident bacteria.

Prebiotics are a special form of nutrient intervention. Instead of providing energy and nutrition directly to the host, prebiotics are metabolized by the GIT bacteria. Many prebiotics selectively encourage the growth of some, but not all, bacterial groups. A number of phytochemicals have been investigated for the ability to regulate the composition and metabolic activities of the GIT bacteria. Although fermentable fibers have received most of the attention and are the subject of the remainder of this contribution, other phytochemicals, such as tea polyphenols, have shown promise (Narisawa and Fukaura, 1993).

## FERMENTABLE FIBERS

The component of a diet that is resistant to hydrolysis by vertebrate digestive enzymes is considered to be "fiber." Supplementing a diet with fiber increases stool volume and weight, reduces residence time of digesta, and has been associated with a lower risk of colon cancer, apparently due to reduced exposure to carcinogens (Cummings et al., 1992). Adding fermentable fibers to enteral diets elicits dramatic benefits, such as stimulating the growth, architecture, and functions of the small intestinal mucosa (Chinery et al., 1992), reducing the risk of bacterial translocation and septicemia (Spaeth et al., 1990), and, in conjunction with other ingredients, enhancing synthesis and secretion of immune modulators (Campbell et al., 1997a).

Traditionally, fibers are classified based on solubility in water. More recently, fibers are characterized on how well they can be metabolized (fermented) by GIT bacteria. The principal metabolites of fermentation are short chain fatty acids (SCFAs) that are available to the host for energy. The proportions of the different fatty acids that are produced vary among the types of fibers and are also dependent on the assemblage of bacteria (Cummings and Macfarlane, 1991; Campbell et al., 1997b; Buddington and Sunvold, 1998). Although fermentation of fiber is usually considered to occur mainly in the colon, it can be detected throughout the entire GIT, including the stomach of monogastric species (Argenzio and Southworth, 1974), with fermentation in more proximal regions thought to be substantial (McBain and Macfarlane, 1997).

Fermentable fibers increase densities of lactic acid bacteria and reduce the number of Enterobacteriaceae, which include most pathogens (Rowland and Tanaka, 1993; Wang and Gibson, 1993), such as *Salmonella* (Bovee-Oudenhoven et al., 1997), as well as other groups that can be pathogenic (Terada et

al., 1994). The increased proportions of lactic acid-producing bacteria are also associated with reduced translocation of *Candida albicans* from the GIT to the mesenteric lymph nodes (Berg et al., 1993), lower bioavailability of some toxins and carcinogens (Zhang and Ohta, 1993), and improved health of patients with chronic inflammatory bowel disease (Teramoto et al., 1996).

The fatty acids produced by fermentation influence the luminal environment. They also influence GIT structure and functions (Murray, 1990) that may be related to induced expression of early response genes (Tappenden and McBurney, 1998). This includes enhanced colonic epithelial cell proliferation and protein synthesis (Marsman and McBurney, 1996), increased mucosal mass and functional properties (Howard et al., 1995), increased size of other digestive organs (Hoshi et al., 1994), and the secretion of glucagon-like peptides and probably other biologically active substances that stimulate growth and functional properties of the proximal small intestine (McBurney et al., 1998). The increased densities of lactic acid-producing bacteria in response to dietary fibers also have "global" influences, such as being associated with decreased serum cholesterol (Kishimoto et al., 1995). The health benefits of the lactic acid bacteria and fermentable fibers have stimulated interest in the development of symbiotics, which are supplements containing both fermentable fiber and prebiotics (Schaafsma et al., 1998).

There is a wide diversity of fermentable fibers that are being considered as dietary supplements. The fructooligosaccharides (FOSSs) have received most of the attention, but some of the others that have been examined include galactosylsucrose (Kumemura et al., 1992), lactulose (Terada et al., 1994; Hara et al., 1994), and xylosylfructoside (Hoshi et al., 1994). Although the above fermentable fibers are considered to be safe for consumption and provide health benefits, each has a maximum tolerated dose above which diarrhea results, apparently because of osmotic effects.

FOSSs are  $\beta 1-2$  linked polymers of fructose with one terminus being either fructose or glucose (Roberfroid et al., 1993) and varying degrees of polymerization. FOSSs are present in a wide diversity of plants (Hidaka et al., 1986; Van Loo et al., 1995) and, like other fermentable fibers, are not digested by vertebrate enzymes (Oku et al., 1984). Most of the ingested FOSSs transit the small intestine (>80%) and are almost completely fermented in the colon (Molis et al., 1996; Rumessen et al., 1990), where there is an interesting, but poorly understood, relationship with dietary calcium (Rémésy et al., 1993). FOSSs are metabolized by numerous bacteria, but of relevance to health, they are preferred substrates for lactic acid bacteria and selectively stimulate proliferation of the *Bifidobacteria* (Gibson et al., 1995). However, there are differences among the various species of *Bifidobacteria* in the ability to metabolize and respond to FOSSs of varying chain lengths (McKellar and Modler, 1989). In contrast to the *Bifidobacteria*, *Clostridia* spp. and *E. coli* have little if any ability to utilize FOSSs.

Fermentation of FOSs produces SCFAs, hydrogen gas, and other metabolites. The SCFAs are available to the host, allowing a portion of the energy associated with FOS to be used by the host (Tokunaga et al., 1989). Supplementing the diet with FOSs as well as other fermentable fibers, initially causes flatulence. Chronic consumption can lead to adaptation of the colonic bacteria, but the response varies among individuals and may not result in diminished production of hydrogen or improved tolerance (Stone-Dorshow and Levitt, 1987; Briet et al., 1995).

The health benefits caused by supplementing a diet with FOS are similar to those known for other fermentable fibers. In addition, FOSs are known to increase true calcium absorption (Morohashi et al., 1998). The following sections describe how FOS and other fermentable fibers can be used as tools to manage the GIT throughout the life history, in health and in disease states.

## **MANAGING THE MATURE GIT**

Many ecologists are interested in understanding if and how mature ecosystems respond to inputs. Many nutritionists are addressing the same questions with the mature GIT ecosystem of adult humans and other mammals. FOSs are now recognized as a useful tool that can be used to manage the bacterial assemblages resident in the GIT ecosystem. Feeding adult humans a diet supplemented with FOSs increases the proportion of the fecal flora represented by lactic acid-producing bacteria, with the responses directly related to dose (Gibson and Roberfroid, 1995). There are concurrent reductions in the relative densities of potential pathogens, with similar findings from animal models (Bailey et al., 1991; Waldroup et al., 1993).

The changes in bacterial assemblages induced by FOS are associated with lower activities of some reductive enzymes that are correlated with increased risk of colon cancer (McConnell and Tannock, 1993; Buddington et al., 1996). Other health benefits include enhanced immunity, as is evident from increased growth of enteric lymphoid tissue (Pierre et al., 1997) and reduced incidence of colon tumors, improved serum lipids, and changes in insulin secretion and glucose homeostasis (Rumessen et al., 1990; Luo et al., 1996).

The higher densities of lactic acid-producing bacteria are also associated with greater mucosal mass and surface area and increased nutrient transport functions in dogs (Buddington et al., 1998) and mice (our unreported data). As a consequence, the absorptive capacities of the GIT are increased.

## **MANAGING THE DEVELOPING GIT**

The GIT of neonates is markedly different from that of adults. Although

the GIT is sterile at birth, it is rapidly colonized by organisms present in the external environment; by 12 hours after birth, densities of bacteria in stool samples are comparable to those of adults (Swords et al., 1993). The importance of the initial inoculum is evident from the different bacterial assemblages that are present in the stools of infants delivered vaginally and thereby exposed to maternal fecal and vaginal bacteria and those by Caesarian section.

Postnatal changes in the GIT bacteria provide an interesting opportunity to study developmental ecology on a small, though complex, scale (Mackie et al., 1998). Acquisition of the adult assemblages of bacteria requires at least several months and involves a series of successional stages (Conway, 1996; Gibson and Roberfroid, 1995; Swords et al., 1993). Initially, aerotolerant groups dominate, but the production of organic acids and the utilization of oxygen lead to a reduced, anaerobic environment that allows anaerobic forms to proliferate and eventually dominate. The metabolic characteristics of the bacteria also take time to develop, with production of short chain fatty acids not increasing appreciably in pigs until about the third week after birth (Murray et al., 1987).

Diet is an important determinant of bacterial populations during infancy. This is evident from the higher densities of lactic acid bacteria in the stool of breast-fed infants compared to those who receive formula (Mackie et al., 1998). In light of the possible health benefits provided by the higher densities of lactic acid-producing bacteria, there is great interest in identifying compounds that can be added to formula and that will increase the densities of Bifidobacteria and Lactobacilli. The FOSs have received the greatest attention, and, similar to results for human adults, densities of Bifidobacteria are higher in suckling pigs fed a milk replacer supplemented with short chain FOSs (our unpublished data).

Although higher densities are apparent in all regions of the GIT, they are more pronounced in the upper small intestine and include both luminal and mucosal bacteria. It is possible that the general lack of FOSs influences reported for clinical studies with human infants might be related to the bacteriologic analysis of stools. Similarly, if river ecologists are restricted to the analysis of water samples at the mouths of rivers, they may very well miss important events and processes occurring upstream. At the present time, the health benefits associated with adding FOSs to formulas for infants and milk replacers for companion animals and species of agricultural importance are unknown. However, recent studies with newly hatched quail show that the Bifidobacteria provide resistance against necrotizing enterocolitis (Butel et al., 1998). Furthermore, the addition of oligofructose to the diet fed to newly hatched chicks selectively stimulates proliferation of the Bifidobacteria and other lactic acid-producing bacteria and thereby provides protection against necrotizing enterocolitis (Catala et al., 1998).

Diet plays another important role at the time of weaning, which is a critical

period when maternal antibodies are no longer available. In addition to direct influences on the bacterial assemblages, the transition from milk to a solid diet is associated with changes in GIT structure and functions (Buddington, 1994), effectively altering the physical and chemical environments. There is limited evidence that supplementing the weaning diet with FOSs stimulates proliferation of lactic acid bacteria, reduces the incidence of diarrhea and other digestive problems commonly seen at weaning, and improves health and feed efficiency (Fukuyasu et al., 1987).

## **MANAGING THE SENESCENT GIT**

Senescence is accompanied by declines in several physiological functions that are associated with digestion and is often accompanied by problems of defecation (e.g., constipation). Notable is the decreased production of gastric and pancreatic secretions. There can be concurrent changes in the densities and composition of the bacteria resident in the different regions of the GIT, with declines in the densities of lactic acid bacteria (Toskes, 1993). These changes can be detrimental and include overgrowth in the small intestine.

The bacteria present in the senescent GIT are responsive to the addition of fermentable fiber, as demonstrated by the first studies showing the beneficial influences of FOSs. Specifically, densities of *Bifidobacteria* increased when the diets of elderly patients in retirement homes were supplemented with FOS (Mitsuoka et al., 1987), with similar findings reported for other fermentable fibers. Collectively, these findings show that the senescent GIT and the resident bacteria are responsive to dietary inputs (Kumemura et al., 1992). However, it is uncertain if the changes in the GIT bacteria stimulate GIT growth and digestive functions.

## **MANAGING RECOVERY OF THE GIT**

The structure and functions of a river ecosystem are partly determined by the hydrologic regime. Disturbances caused by floods of intermediate magnitude and frequency are considered to be critical for maintaining the diversity of organisms in a river by slowing competitive exclusion and providing open microenvironments (reviewed by Buddington and Weiher, 1999). Whereas seasonal, tidal, and other small floods are needed to maintain the diversity, frequent large floods or the complete lack of floods reduces diversity and the functional elements.

Meals, which represent small scale, periodic "floods," are critical for maintaining the normal GIT bacterial assemblages. In contrast, diarrhea, which can be considered as a large magnitude disturbance, disrupts the structure and

functions of the GIT (Fagundes-Neto et al., 1997; Guandalini, 1988) and alters the species assemblages in the different regions (Oli et al., 1998). During and immediately after diarrhea, aerobic bacteria are displaced such that they are detected at higher densities in the stool (Fagundes et al., 1976) and can be associated with aerobic overgrowth in the proximal small intestine (Bhan et al., 1989). Diarrhea-induced disturbances of the normal GIT bacteria reduce competitive exclusion of pathogens and thereby increase the risk of secondary infections. The administration of certain antibiotics can also disturb the normal bacterial assemblages and, by doing so, allow pathogenic species, such as *Clostridium difficile*, to proliferate (Wilson, 1993) and induce diarrhea and other GIT disease states.

After a large-scale disturbance, it is desirable to return an ecosystem to normalcy. After a disturbance, species with the shortest generation times will recover faster. Unfortunately, in the GIT, pathogens tend to have shorter generation times than bacteria considered to be beneficial (Oli et al., 1998). Two approaches can be used to accelerate the recovery of the GIT ecosystem after diarrhea. Probiotics can be administered to effectively "seed" the newly opened microenvironments with beneficial bacteria and thereby reduce space and nutrients for pathogens. The second approach is the use of prebiotics, which encourage the proliferation of beneficial bacteria that are already resident in and adapted to the GIT, and are perhaps more effective than probiotic approaches at excluding pathogens. We have already shown that the addition of a short chain FOSs (0.5%) to an oral electrolyte solution accelerates recovery of the beneficial bacteria in the GITs of pigs with diarrhea induced by cholera toxin (Oli et al., 1998). The same study showed that the intestinal mucosal mass of pigs recovered faster when FOSs were added to the oral electrolyte solution, but additional work is needed to determine if GIT functions also recovered faster.

## PERSPECTIVES

River ecosystems change over geological time, whereas the GIT ecosystems change during the life history. Both are susceptible, and somewhat dependent, on disturbances for maintaining normal structure and functions. The challenges, and questions, facing individuals managing either ecosystem are several-fold.

The first problems are to identify what components of an ecosystem should be managed—the physical, chemical, or biotic components—and the most appropriate method(s) of management. A second concern is locating the site of management. Although management of an entire ecosystem is desirable, it is often not practical or feasible. As a result, management efforts must be targeted to a specific region(s). Third, in some situations, it will be necessary

to establish when to manage. For example, it is unknown if efforts to "improve" the GIT ecosystem should occur from birth to death or be limited to specific stages of development. And finally, managers of ecosystems must always be aware of the limitations and possible complications of management approaches.

Phytochemicals, particularly fermentable fibers, appear to be very useful tools for managing the GIT ecosystem. When used in moderation, they cause changes in the species and metabolic characteristics of the GIT bacterial assemblages that are considered to be beneficial. The changes are associated with improved GIT structure and functions, and include increased resistance to diseases. Further research is needed to obtain insights about how to optimize the use of phytochemicals as tools to manage the GIT ecosystem.

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# **Phytoantimicrobial (PAM) Agents as Multifunctional Food Additives**

A. S. NAIDU

## **INTRODUCTION**

**F**ood preservation dates back to prehistoric times and has become refined into an art in various cuisines around the world. The potential benefits of edible plants as well as their phytochemicals in food preservation and improvement of organoleptic qualities of certain traditional foods have been practiced for centuries. Ancient Egyptians used spices and oils for preventing food spoilage as well as for embalming the dead. The therapeutic use of garlic for a variety of ailments, including indigestion, pneumonia, wounds, and infections, were cited by Hippocrates, Pliny, and Virgil. Although ancient civilizations acknowledged the antiseptic and antimicrobial potential of many plant extracts, it was not until recently that the implied phytochemicals were characterized. Advances in molecular separation techniques led to the isolation of various phytoantimicrobial (PAM) compounds. The increased demand for minimally processed, extended shelf-life foods has further revived interest in exploitation of these natural PAM agents.

Effective PAM agents preserve food by various mechanisms including stasis (growth inhibition of microorganisms) and cidal (direct destruction of microorganisms) effects. Certain PAM agents seem to deliver multifunctional physiological benefits to consumers and, therefore, are highly attractive to the health food industry. Because PAM compounds have been in the food supply and consumed for many years, these natural phytochemicals appear to be safe when compared to new synthetic preservatives. This chapter will elucidate a myriad of PAM agents in nature and a possible role of these compounds as additives in the enhancement of shelf life and safety of foods.

## PAM FROM OILS

The storage of yogurt under olive oil has been practiced since Biblical times, and it is assumed that the oil has a preservative role. Recently, a number of potential PAM compounds have been isolated from olives and virgin olive oil, and among these are polyphenols and glycosides. Some of these PAM are effective against lactic acid bacteria. The compounds identified are tyrosol, *p*-hydroxyphenylacetic acid, *p*-coumaric acid, and ferulic acid (Keceli et al., 1998).

Lachowicz et al. (1998) examined essential oils from five varieties of *Ocimum basilicum* L. plants (anise, bush, cinnamon, dark opal, and dried basil) for antimicrobial activity against a wide range of foodborne gram-positive and -negative bacteria, yeasts, and molds. All five essential oils showed antimicrobial activity against most of the organisms tested, except *Flavimonas oryzihabitans* and *Pseudomonas* species. Synergistic effects were observed between anise oil, low pH (4.2), and salt (5% NaCl). Anise oil demonstrated antimicrobial effects in tomato juice medium and inhibited the growth of *Lactobacillus curvatus* and *Saccharomyces cerevisiae*. Wan et al. (1998) also examined the antimicrobial activity of basil essential oils, including basil sweet linalool and basil methyl chavicol against a range of gram-positive and gram-negative bacteria, yeasts, and molds. Both essential oils inhibited most of the microorganisms except *Clostridium sporogenes*, *Flavimonas oryzihabitans*, and three species of *Pseudomonas*. The minimal inhibitory concentration (MIC) of chavicol against *Aeromonas hydrophila* and *Pseudomonas fluorescens* was 0.125 and 2% (v/v), respectively.

Smith-Palmer et al. (1998) tested the antimicrobial properties of 21 plant essential oils and two essences against five important food-borne pathogens including *Campylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The oils of bay, cinnamon, clove, and thyme were the most inhibitory, each having a bacteriostatic concentration of 0.075% or less against all five pathogens. In general, gram-positive bacteria were more sensitive to inhibition by plant essential oils than the gram-negative bacteria. *Campylobacter jejuni* was the most resistant of the bacteria investigated to plant essential oils, with only the oils of bay and thyme having a bactericidal concentration of less than 1%. *L. monocytogenes* was extremely sensitive to the oil of nutmeg. A concentration of less than 0.01% was bacteriostatic and 0.05% was bactericidal, but when the temperature was reduced to 4 degrees, the bacteriostatic concentration was increased to 0.5% and the bactericidal concentration to >1%.

Patnaik et al. (1997) tested the antimicrobial activity of five aromatic constituents of essential oils (cineole, citral, geraniol, linalool, and methol) against 18 bacteria (including gram-positive cocci and rods and gram-negative rods) and 12 fungi (three yeast-like and nine filamentous). Linalool was the

most effective and inhibited 17 bacteria, followed by cineole, geraniol (each of which inhibited 16 bacteria), menthol, and citral aromatic compounds, which inhibited 15 and 14 bacteria, respectively. Against fungi, the citral and geraniol oils were the most effective (inhibiting all 12 fungi), followed by linalool (inhibiting 10 fungi), cineole, and menthol (each of which inhibited seven fungi) compounds.

Shcherbanovsky and Kapelev (1975) reported the antimicrobial activity of 25 volatile oils from aerial parts and seeds of dill (*Anethum graveolens* L.) against yeast *Saccharomyces vini* and *Lactobacillus buchneri*. Yousef and Tawil (1980) evaluated the bacteriostatic and fungistatic activities of 22 volatile oils, wherein, the cinnamon oil showed the highest activity against the tested bacteria and fungi.

The essential oil from herb of *Ducrosia anethifolia* (DC.) Boiss. consists mainly of aliphatic compounds (Janssen et al., 1984). Alpha-pinene, myrcene, and limonene are main components of the hydrocarbons present in the oil, while *N*-decanal, *N*-dodecanal, *N*-decanol, *trans*-2-dodecenal, and *cis*-chrysanthenyl acetate are the major oxygen-containing constituents. The oil and the main oxygen-containing aliphatic components show a potent antimicrobial activity against gram-positive bacteria, yeast, and dermatophytes.

Aromatic plants from the Labiatae family (*Thymus vulgaris*, *Ocimum gratissimum*), the Myrtaceae family (*Eugenia caryophyllata*, *Melaleuca viridiiflora*), and the Compositae family (*Helichrysum lavanduloides*, *H. bracteiferum*, *H. gymnocephalum*, *Psiadia altissima*) show antimicrobial activity against enteropathogenic and food spoilage organisms (Ramanoelina et al., 1987). Three oils from *Thymus vulgaris*, *Ocimum gratissimum*, and *Eugenia caryophyllata* demonstrated broad-spectrum activity. The essential oil of *Melaleuca viridiiflora* also had a high inhibitory effect, especially on gram-positive bacteria.

Essential oil from *Achillea fragrantissima* exerts a cidal effect on several gram-positive and gram-negative bacteria as well as on *C. albicans*. The active PAM compound was identified as terpinen-4-ol (Barel et al., 1991). Essential oils from *Cedronella canariensis* (L.) W. et B. inhibit respiratory tract pathogens *Bordetella bronchiseptica* and *Cryptococcus albidus* (Lopez-Garcia et al., 1992). The essential oil from the leaves of *Hoslundia opposita* contains largely the sesquiterpenes and sesquiterpene alcohols. These PAM compounds show significant activity against *Aspergillus niger*, *Acinetobacter calcoaceticus*, *Brochothrix thermosphacta*, and *Flavobacterium suaveolens* (Gundidza et al., 1992). Essential oils from *Satureja montana* L., *Rosmarinus officinalis* L., *Thymus vulgaris* L., and *Calamintha nepeta* (L.) Savi demonstrate potent antimicrobial and fungicidal activities (Panizzi et al., 1993). Camphor and camphene are the major essential oil constituents of *Piper angustifolium* Lam. These PAM compounds are bacteriostatic and fungistatic against *Trichophyton*

*mentagrophytes*, *P. aeruginosa*, *C. albicans*, *Cryptococcus neoformans*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *E. coli* (Tirillini et al., 1996).

The volatile oil of *Ducrosia ismaelis* Asch. is a light yellow volatile liquid with a strong aromatic odor and a specific gravity of 0.9573 (Al-Meshal, 1986). Spectrometry studies revealed the presence of free alcohols, alkenes and highly conjugated alkenes, aromatic functions, alicyclic structures, and cyclic ketones. The pharmacological studies of this oil showed a highly significant and dose-dependent central nervous system depressant and marked neuromuscular blocking actions. Experiments on smooth muscles and heart show a parasympatholytic activity. It also exhibits significant antimicrobial activity against *S. aureus*, *Bacillus subtilis*, and *C. albicans*.

Tea-tree oil (an essential oil of the Australian native tree *Melaleuca alternifolia*) has long been regarded as a useful topical antiseptic agent in Australia and has been shown to have a variety of antimicrobial activities; however, only anecdotal evidence exists for its efficacy in the treatment of various skin conditions. Bassett et al. (1990) conducted a single-blind, randomized clinical trial on 124 patients to evaluate the efficacy and skin tolerance of 5% tea-tree oil gel in the treatment of mild to moderate acne when compared with 5% benzoyl peroxide lotion. The results of this study showed that both 5% tea-tree oil and 5% benzoyl peroxide had a significant effect in ameliorating the patients' acne by reducing the number of inflamed and non-inflamed lesions (open and closed comedones), although the onset of action in the case of tea-tree oil was slower.

## PAM FROM SPICES

Scientific evidence on preservation potential of spices emerged early in the 19th century. Chamberland (1887) first reported the antimicrobial activity of cinnamon oil against spores of anthrax bacilli. Grove (1918) observed the ability of aqueous and alcoholic extracts of ground cinnamon to preserve tomato sauce. Prasad and Joshi (1929) developed a method in India for preserving native fruits with ground cloves and salt. Fabian et al. (1939) found that cinnamon inhibits microbial growth at a 1:50 dilution (extract of 10 g in 100 ml of water); and cloves inhibit *Bacillus subtilis* at 1:100 and *Staphylococcus aureus* at 1:800 dilutions, respectively.

Conner and Beuchat (1984) reported that an oleoresin of cinnamon was extremely inhibitory against eight yeasts, i.e., *Candida lipolytica*, *Debaryomyces hansenii*, *Hansenula anomala*, *Kloeckera apiculata*, *Lodderomyces elongisporus*, *Rhodotorula rubra*, *S. cerevisiae*, and *Torulopsis glabrata*. Essential oil of clove dispersed (0.4% v/v) in a concentrated sugar solution had a marked germicidal effect against various bacteria and *C. albicans* (Briozzo et al., 1989). *S. aureus* (five strains), *Klebsiella pneumoniae*, *P. aeruginosa*,

*Clostridium perfringens*, and *E. coli* inoculated at a level of  $10^7$  cfu/ml and *C. albicans* (inoculum  $4.0 \times 10^5$  cfu/ml) were killed (>99.9%) after two to seven minutes in broth supplemented with 63% (v/w) of sugar and containing 0.4% (v/w) of essential oil of clove. Presence of organic matter (i.e., human or bovine serum) did not impair its antimicrobial activity. Sugar was not necessary for the antimicrobial activity of clove oil, but the concentrated sugar solution provided a good vehicle for obtaining uniform oil dispersion that is relatively stable for certain practical applications.

Antimicrobial activity of cinnamon, allspice, and cloves is attributed to eugenol (2-methoxy-4-allyl phenol) and cinnamic aldehyde, which are major constituents of the volatile oils of these spices. Cinnamon contains 0.5 to 1.0% volatile oil, which contains 65 to 75% cinnamic aldehyde and 8% eugenol. Allspice contains up to 4.5% volatile oil, of which 80% is eugenol. Clove buds have an average essential oil content of 17% that is 93 to 95% eugenol (Farrell 1985).

Oregano, savory, and thyme demonstrate antifungal activity. Terpenes carvacrol, *p*-cymene, and thymol are the major volatile components of oregano, thyme, and savory and likely account for the antimicrobial activity. The essential oil of oregano contains up to 50% thymol; thyme has 43% thymol and 36% *p*-cymene; and savory has 30 to 45% carvacrol and 30% *p*-cymene (Farag et al., 1989).

Spice oils and extracts of sweet marjoram, laurel, pimiento (Chile), coriander, anise, carvone, peppermint, caraway, cardamom, cumin, fennel, celery, dill, and mustard also exhibit antimicrobial activity (Marth, 1966). Rosemary spice extract inhibits the growth of *Salmonella typhimurium* and *S. aureus* (Farbood et al., 1976). Rosemary or sage at a concentration of 0.3% inhibited the proliferation of 20 food-borne gram-positive organisms, whereas, at 0.5% concentration, these substances are considered bactericidal (Shelef et al., 1980). The inhibitory effects of rosemary and sage were attributed to their terpene fraction comprised of borneol, cineole, pinene, camphene, camphor (all rosemary), and thujone (sage).

Turmeric was shown to inhibit a variety of bacteria, including *Bacillus cereus*, *S. aureus*, *E. coli*, and *Lactobacillus plantarum* (Bhavani Shankar and Sreenivasa Murthy, 1979). Alcoholic extracts of rosemary and turmeric could inhibit germination, growth, and toxin production by *Clostridium botulinum* at 500 ppm concentration (Huhtanen, 1980), whereas, nutmeg, curry powder, mustard, black pepper, and sassafras could moderately inhibit *V. parahaemolyticus* (Beuchat, 1976). The spice *Aframomum danielli* on a wet weight basis with a moisture content of 10.5%, protein content of 8.2% (dry matter basis) could inhibit the growth of *Salmonella enteritidis*, *Pseudomonas fragi*, *P. fluorescens*, *Proteus vulgaris*, *Streptococcus pyogenes*, *S. aureus*, *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus*, and *A. niger*. The MIC determined for

*Klebsiella pneumoniae* and *P. aeruginosa* was one in 32 whilst the MIC for *S. aureus* was one in 8000 (Adegoke and Skura, 1994).

## PAM FROM FRUIT AND VEGETABLES

The antimicrobial activity of many of the vegetable extracts may be due in part to the presence of low-molecular-weight antimicrobial compounds, "phytoalexins," produced by plant tissues in response to stress, trauma, or infection (Beuchat and Golden, 1989). Kurosaki and Nishi (1983) isolated 6-methoxymellein as a phytoalexin produced by carrot roots, which showed the broad-spectrum ability to inhibit growth of various molds, yeasts, and bacteria. The 6-methoxymellein inhibited yeasts by interacting with membrane components and disrupted membrane function in a nonspecific manner (Amin et al., 1988). Volatile components of carrot contain significant levels of several oxygenated acyclic and monocyclic terpenoids, which are also antimicrobial (Batt et al., 1983). Aqueous extract of carrot could inhibit growth multiplication of *L. monocytogenes*, and this activity is dependent on PAM concentration, pH, and presence of sodium chloride (Beuchat et al., 1994). Purified methol extracts of carrots are bactericidal against *Leuconostoc mesenteroides*, *S. aureus*, *L. monocytogenes*, *E. coli*, *P. fluorescens*, and the yeast *Candida lambica* at concentrations ranging from 55 to 220 mg/ml (Babic et al., 1994). Phytochemicals dodecanoic (lauric) acids, methyl esters of dodecanoic (monolaurin) acid, and pentadecanoic acid have been identified as the potent PAM compounds of carrot.

Interactions of monolaurin, eugenol, and sodium citrate on the growth of six organisms including common meat spoilage (*Lactobacillus curvatus*, *Lactobacillus sake*, *Leuconostoc mesenteroides*, *Brochothrix thermosphacta*) and pathogenic (*Escherichia coli* O157:H7 and *L. monocytogenes*) organisms were investigated (Blaszyk and Holley, 1998). The combinations of 100 to 250 ppm monolaurin with 500 and 1000 ppm eugenol and 0.2 and 0.4% sodium citrate were more effective than each component separately. More than one combination prevented detectable growth of each organism. Lactic acid bacteria and *E. coli* O157:H7 were most resistant, and *L. monocytogenes* and *B. thermosphacta* were most sensitive. The presence of sodium citrate was necessary to yield potent inhibition of *L. curvatus* and *L. sake* growth by the monolaurin and eugenol combinations.

Methanolic extracts of sweet potato, cabbage, radishes, green beans, beets, cauliflower, peas, peppers, rhubarb, spinach, brussels, sprouts, and tomatoes demonstrate antimicrobial activity (Marth, 1966). In tomatoes, tomatine, a glycosidal alkaloid, was identified as the active component. A cinnamic acid derivative of white potatoes inhibited aflatoxigenic *Aspergillus parasticus* (Swaminathan and Koehler, 1976). Similar inhibition of aflatoxin formation

by *A. parasticus* was also reported with carrot root extract (Batt et al., 1983). Volatile terpenoid components of the oil from carrot seed also seem to inhibit aflatoxin formation.

Isothiocyanate (ITC) phytochemical derivatives from glucosinolates of Cruciferae or mustard family (cabbage, kohlrabi, brussels sprouts, cauliflower, broccoli, kale, horseradish, mustard, turnips, and rutabaga), are potent PAM agents. ITC compounds are inhibitory to fungi, yeasts, and bacteria in the range of 0.016 to 0.062 µg/ml in the vapor phase or 10 to 600 µg/ml. The mechanism of ITC antimicrobial activity seems to involve enzymes attacking disulfide bonds via thiocyanate anion reaction and inactivate sulphydryl enzymes. The ITC may also act as uncouplers of oxidative phosphorylation. Despite very low sensory thresholds, ITC compounds could be useful as food antimicrobials due to their low inhibitory concentrations. Shofran and co-workers (1998) recently suggested a possible application of allyl ITC as a natural preservative for non-acidified, refrigerated pickled vegetables. Allyl ITC seems particularly effective as a PAM against Enterobacteriaceae, with rate of bacterial survival significantly reduced at 30 ppm.

## PAM FROM HERBS

Therapeutic properties of herbs have been recognized since antiquity. The advent of molecular pharmacology has paved the way for many herbal PAMs into modern medicine. The rapid international growth of the natural products market and the proactive stance of the consumer toward health foods and nutraceuticals have opened unlimited possibilities for herbal PAMs as food additives.

## SAPONINS

Ethanol and aqueous extracts of *Calliandra portoricensis* leaves contain saponins, tannins, flavonoids, and glycosides (Aguwa and Lawal, 1988). Both extracts inhibit ulcerogenic effects of pylorus ligation and stress in rats. The anti-ulcer effects of the aqueous extract were always more significant than that of the ethanolic extract. This indicates that the higher content of the saponins and/or tannins of the leaf extract may be responsible for the anti-ulcer effects. The leaf extracts also inhibit *E. coli*, *S. aureus*, and *Streptococcus faecalis*.

Extracts of the desert plant *Yucca shidigera* were suggested for their possible benefit in ruminal fermentation (Wallace et al., 1994). Inclusion of *Y. shidigera* extract (1%, vol/vol) in the growth medium of the rumen bacterium *Streptococcus bovis* extended its lag phase, while growth of *Butyrivibrio fibrisolvens* was inhibited. The growth of *Prevotella ruminicola* was stimulated, and that

of *Selenomonas ruminantium* was unaffected. Protozoal activity, as measured by the breakdown of <sup>14</sup>C-leucine-labelled *S. ruminantium* in rumen fluid incubated *in vitro*, was abolished by the addition of 1% extract. The antimicrobial activities were unaffected by precipitating tannins with polyvinylpyrrolidone, but a butanol extract, containing the saponin fraction, retained its antibacterial and antiprotozoal effects. Saponins from other sources were less effective against protozoa than *Y. shidigera* saponins. *Y. shidigera* extract, therefore, appears unlikely to influence ammonia concentration in the rumen directly, but its saponins have antimicrobial properties, particularly in suppressing ciliate protozoa, which may prove beneficial to ruminal fermentation and may lead indirectly to lower ruminal ammonia concentrations.

Ethanol and aqueous extracts of *Bridelia ferruginea*, at a final concentration of 5 mg/ml, produce *in vitro* antimicrobial activities against clinical isolates of *S. aureus*, *C. albicans*, *S. epidermidis*, *E. coli*, *Streptococcus lactis*, *Proteus vulgaris*, *Proteus mirabilis*, *Streptococcus pyogenes*, and *Klebsiella sp.* (Irobi et al., 1994). Preliminary phytochemical analysis of the plant extracts showed the presence of phenols and tannins.

Tea-leaf saponin from leaves of *Camellia sinensis* var *sinensis* show high antimicrobial activity against pathogenic dermal fungi, and its MIC value for *Microsporum audouinii* was 10 µg/ml (Sagesaka et al., 1996). On the other hand, tea-leaf saponin inhibited rat paw edema induced by carrageenan in a dose-dependent manner. Activation of hyaluronidase, one of the enzymes involved in inflammatory reactions, was inhibited by tea-leaf saponin. It was also found that tea-leaf saponin antagonized the action of leukotrien D4, one of the chemical mediators of inflammatory reactions.

Organic and aqueous solvent extracts of *Arctotis auriculata Jaca*, *Eriocephalus africanus L.*, *Felicia erigeroides DC.*, and *Helichrysum crispum (L.) D. Don* demonstrate selective antimicrobial activities (Salie, et al., 1996). Organic extracts of *A. auriculata* and *H. crispum* inhibit the growth of *Mycobacterium smegmatis*. The same extracts, together with organic extracts of *F. erigeroides*, were active against *P. aeruginosa*. Antifungal activities against *C. albicans* were exhibited by organic extracts of *E. africanus*, *F. erigeroides*, and *H. crispum*. Organic extracts of *A. auriculata* and *E. africanus*, as well as the aqueous extract of the latter plant, were also active against *S. aureus*.

Extracts of foliage from African multipurpose trees *Acacia aneura*, *Chamaecytisus palmensis*, *Brachychiton populneum*, *Flindersia maculosa*, *Sesbania sesban*, *Leucaena leucocephala*, and *Vernonia amygdalina* inhibit rumen protozoa and bacteria (Newbold et al., 1997). The antimicrobial effects were mild except for *S. sesban*, which was highly toxic to rumen protozoa *in vitro*, and *A. aneura*, which was toxic to rumen bacteria. The antiprotozoal factor in *S. sesban* was apparently associated with the fraction of the plant containing saponins. When *S. sesban* was fed to sheep, protozoal numbers were reduced by 60% after day 4, but the population recovered after day 10. *In vitro*

experiments demonstrated that washed protozoa from later times were no more resistant to *S. sesban* than on initial exposure, suggesting that other microorganisms, probably the bacteria, adapted to detoxify the antiprotozoal agent. Thus, *S. sesban* may be useful in suppressing protozoal and, thereby, improving protein flow from the rumen, but only if the bacterial metabolism of the antiprotozoal factor can be avoided.

## FLAVONOIDS

The antimicrobial activities of a number of cytotoxic C-benzylated flavonoids from *Uvaria chamae* were reported (Hufford and Lasswell, 1978). The MIC values of these flavonoids and some of their derivatives against *S. aureus*, *Bacillus subtilis*, and *Mycobacterium smegmatis* compare favorably with those of streptomycin sulfate.

The antimicrobial activity of extracts and constituents of *Gomphrena martiniana* and *Gomphrena boliviiana* (Amaranthaceae) were evaluated against 20 microorganisms, including gram-positive and gram-negative bacteria, spore-forming gram-positive bacteria, an acid-fast bacterium, a fungus, and two yeasts (Pomilio et al., 1992). Fractionation of petroleum ether extract yielded five 5,6,7-trisubstituted flavones that were highly inhibitory against *Mycobacterium phlei* (MIC 15, 20, and 75 µg/ml) similar to commercial bactericides.

Aqueous and ethanol extracts (10–200 mg/ml) as well as saponin, flavonoid, resin, and essential oil of the plant *Thymus capitatus* (10–5000 µg/ml) inhibited the growth of several bacteria and fungi (Kandil et al., 1994).

The methanol extracts of the leaves and stem bark of four Bignoniaceae plants, *Jacaranda mimosifolia* D. Dol., *Tecoma stans* Linn. *Tabebuia rosea* (Bertol) D.C., and *Crescentia cujete* Linn., elicit antimicrobial activity against a wide range of gram-positive and gram-negative bacteria and fungi (Binutu and Lajubutu, 1994). However, methanol extracts of *Tecoma stans* leaves were effective against only *C. albicans*. Preliminary phytochemical screening of these plants revealed the presence of tannins, flavonoids, alkaloids, quinones, and traces of saponins.

The total extract and fractions with different solvents, obtained from leaves of *Tagetes minuta*, show several degrees of antimicrobial activity against gram-positive and gram-negative microorganisms. The same fractions were inactive against *Lactobacillus*, *Zymomonas*, and *Saccharomyces* species. The major component of the extract, quercetagetin-7-arabinosyl-galactoside, also showed significant antimicrobial activity (Tereschuk et al., 1997).

Li et al. (1997) reported a methanol extract of *Ceanothus americanus* with potent antimicrobial activity against selected oral pathogens. Further analysis revealed three triterpenes (ceanothic acid, 27-hydroxy ceanothic acid, and ceanothetic acid) and two flavonoids (maesopsin and maesopsin-6-O-glucoside) as PAM compounds. Ceanothonic acid and ceanothetic acid demonstrated

growth inhibitory effect against *Streptococcus mutants*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, and *Prevotella intermedia* with MICs ranging from 42 to 625 µg/ml.

The antimicrobial properties of the resinous exudates from twigs and leaves of four Chilean species of *Pseudognaphalium*: *P. viravira*, *P. robustum*, *P. heterotrichium*, and *P. cheiranthifolium* against six gram-negative bacteria and five gram-positive bacteria were reported (Mendoza et al., 1997). The antimicrobial activity correlated with the presence in the resinous exudate of ent-16-kauren-19-oic acid and to a lesser extent with the presence of ent-9(11),16-kauradien-19-oic.

The antimicrobial activity of honey against 21 types of bacteria and two types of fungi was reported (Wahdan, 1998). Two important classes of PAM, the flavonoids and the phenolic acids, were identified as potent antimicrobials from honey. In this study, two phenolic acids (caffeic acid and ferulic acid) were extracted from honey and were identified as PAM.

## ALKALOIDS, TANNINS AND OTHER COMPOUNDS

Antimicrobial activity of julifloricine, an alkaloid isolated from *Prosopis juliflora*, was reported against 40 microorganisms, which included 31 bacteria, two *Candida* species, five dermatophytic fungi, and two viruses (Aqeel et al., 1989). Significant inhibitory effect was noted against gram-positive bacteria. The MIC for *S. aureus*, *S. epidermidis*, *S. citreus*, *Streptococcus pyogenes*, and *Sarcina lutea* was 1 µg/ml and against *Streptococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus lactis*, *Corynebacterium diphtheriae*, *Corynebacterium hofmannii*, and *Bacillus subtilis*, 5 µg/ml. Its effect was compared with those of identical concentrations of benzyl penicillin, gentamicin, and trimethoprim. The inhibitory effect of julifloricine on gram-negative bacteria such as the species of *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Enterobacter*, *Aeromonas*, and *Vibrio* was almost insignificant. Julifloricine as compared to micoanzole was found superior against *C. tropicalis* and responded equally to *C. albicans*. As compared to econazole, it was found less effective against both *C. albicans* and *C. tropicalis*. This alkaloid was found inactive against dermatophytic fungi (up to a dose of 10 µg/ml) and viruses that included herpes simplex 1 and Newcastle disease virus.

Aqueous, petroleum-ether, chloroform, and dichloromethane extracts of both the barks and leaves of *Ziziphus abyssinica* and *Berchemia discolor* are inhibitory to *S. aureus*, *E. coli*, and *C. albicans*. The aqueous extracts showed significant activity against *S. aureus* and *C. albicans* (Gundidza and Sibanda, 1991).

The traditional analgesic and antipyretic Ethiopian drug ‘Dingetegna’ is made of dried root material of *Taverniera abyssinica* A. Rich (Leguminosae). In a screening for nematicidal natural products, “Dingetegna” extracts showed

strong nematicidal activity toward *C. elegans*. Medicarpin and 4-hydroxymedicarpin were isolated as nematicidal constituents from the extracts. In a micro-well plate assay for nematicidal activity, both compounds exhibited an LD<sub>50</sub> of 25 µg/ml toward *C. elegans*. Beside these nematicidal effects, weak cytotoxic and antimicrobial activities were observed (Stadler et al., 1994).

Delipidated soybeans (Glyteer; GL) possess a broad antimicrobial spectrum against bacteria and fungi (Ito et al., 1995). The antimicrobial activity of GL was cidal and more effective against fungi than bacteria. Furthermore, GL had an effect on methicillin-resistant *S. aureus*. Resistance to GL was not induced in broth cultures of *E. coli*, *S. aureus*, *Streptococcus pyogenes*, *C. albicans*, and *Trichophyton mentagrophytes*.

Antimicrobial effect of leaves from tannin-containing plants *A. nilotica* and *A. farnesiana* and their extracts on *Clostridium perfringens*, *E. coli*, and *S. typhimurium* was reported *in vitro* at dilutions of 0.5% and 0.05% (Sotohy et al., 1995). The results revealed that the total soluble polyphenols ranged from 10.3% to 35.5% and the condensed tannins from 0.5% to 8.3% on dry matter base. The antimicrobial effect of the plant material was only observed on *Clostridium perfringens* but not on *E. coli* and *Salmonella typhimurium*. *A. nilotica* leaves destroyed the suspension of *Clostridium perfringens* instantly; however, the leaves showed a delayed effect. Plant extracts were less effective than the raw plant material. *A. nilotica* leaves destroyed the bacterial suspension after 10 minutes only at the concentration of 0.5%, but not at 0.05%.

Two new antimicrobial peptides related to the gamma-thionine family have been isolated by acid extraction from the broad bean *Vicia faba* (Zhang and Lewis, 1997). The extract was separated by ion exchange chromatography, and a fraction showing antibacterial activity was further purified by reverse-phase HPLC. Material from a single HPLC peak was sequenced and revealed the presence of two peptides differing by one amino acid. The peptides were named fabatins. They are 47 amino acids long, have an overall positive charge, and contain eight cysteines that probably form four disulfide bridges characteristic of the gamma-thionins. Fabatins were active against both gram-negative and gram-positive bacteria, but were inactive against the yeasts *Saccharomyces cerevisiae* and *C. albicans*.

Licochalcone A-D and echinatin, retrochalcones isolated from the roots of *Glycyrrhiza inflata*, show antimicrobial activity (Haraguchi et al., 1998). Among them, licochalcone A and C had potent activity against some gram-positive bacteria. These retrochalcones inhibit oxygen consumption in susceptible bacterial cells.

## PAM—THIOSULFINATES FROM GARLIC

*Allium* is a genus of some 500 species belonging to the family Liliaceae.

However, only a few of these are important as food plants, notably onion, garlic, chive, leek, and rakkyo. Such plants have been used for many centuries for the pungency and flavoring value and for their medicinal properties, and, in some parts of the world, for their use also has religious connotations (Fenwick and Hanley, 1985). The juice and vapors of onions, garlic, and horseradish have been evaluated for their antimicrobial activity since the early 1900s. Walker and co-workers (1925) reported the fungistatic properties of garlic/onion juice and vapors. Walton et al. (1936) developed a simple method for evaluating the antimicrobial activity of garlic vapors. An agar plate is inverted, and minced garlic is placed inside the top lid to expose the media to vapors. After exposure for varying lengths of time, the media are streaked with the test strains. Strains, including *B. subtilis*, *Serratia marcescens*, and two *Mycobacterium* species, were inhibited to varying extents according to this method.

## ALLICIN

The antimicrobial activity of garlic (*Allium sativum* L.) was reported by Cavallito and Bailey (1944), and the active component diallylthiosulfinate was named allicin. Stoll and Seebeck (1951) confirmed that the allicin is derived from the alliin-alliinase system. Dankert et al. (1979) examined the crude juices of garlic in an agar diffusion test for their growth inhibitory effect on five gram-negative and three gram-positive bacterial species and two yeast species. All test organisms were inhibited by garlic juice. Addition of complex-forming agents and organic matter to the crude juice reduced its activity on all test organisms. Volatile substances showed a strong inhibitory activity after exposure for eight hours or longer at 23°C or 37°C. Minimal inhibition concentrations determined in a dilution test were found to be high for gram-negative bacteria and low for both yeast species. The D-values of different test organisms in undiluted garlic juice were calculated. *P. aeruginosa* had a very low D-value, while the bacteriostatic concentration was high. This indicates a large concentration exponent of crude garlic juice for this organism. The opposite was found for *S. aureus*. The antimicrobial activity of garlic extract on the oral flora of volunteers was investigated by Elnima et al. (1983). A mouthwash containing 10% garlic in quarter Ringer solution elicited a significant reduction in the number of oral bacteria.

The effect of bacteriostatic concentrations of allicin (0.2 to 0.5 mM) on the growth of *Salmonella typhimurium* revealed a pattern of inhibition characterized by

- (1) A lag of approximately 15 minutes between addition of allicin and onset of inhibition
- (2) A transitory inhibition phase whose duration was proportional to allicin concentration and inversely proportional to culture density

- (3) A resumed growth phase that showed a lower rate of growth than in uninhibited controls
- (4) An entry into stationary phase at a lower culture density.

Whereas DNA and protein syntheses showed a delayed and partial inhibition by allicin, inhibition of RNA synthesis was immediate and total, suggesting that this is the primary target of allicin action (Feldberg et al., 1988).

The aqueous extract of garlic and allicin both show a potent *in vitro* antibacterial activity against isolates of multiple-drug-resistant *Shigella dysenteriae* I, *Shigella flexneri* Y, *Shigella sonnei*, and enterotoxigenic *E. coli* (Chowdhury et al., 1991). The minimum inhibitory concentrations of the aqueous extract and allicin against *Shigella flexneri* Y were 5 and 0.4  $\mu\text{l}/\text{ml}$ , respectively. The two agents also showed potent *in vivo* antibacterial activity against experimental shigellosis in a rabbit model. Oral administration of the two agents completely cured the infected rabbits within three days. On the contrary, four of the five rabbits in the control group died within 48 hours after challenge. The experimental groups were pathogen-free on the second day of treatment. The antibacterial activity against the challenge strain was observed in the sera of the treated rabbits with 30 to 60 minutes of administration of the agents. The LD<sub>50</sub> values of the aqueous extract and allicin in mice were 173.78 ml/kg and 204.17  $\mu\text{l}/\text{kg}$  of body weight, respectively. At the therapeutic dose, the two agents did not show any adverse effects on the standard biochemical profile of blood.

*Helicobacter pylori* is the causative agent of gastric ulcers and is implicated in the etiology of stomach cancer. The incidence of gastric cancer is lower in individuals and populations with high allium vegetable intakes. Standard antibiotic regimens against *H. pylori* are frequently ineffective in high-risk populations. Wong et al. (1996) reported the inhibitory activity of garlic extract on *H. pylori*. Sivam et al. (1997) investigated the role of allium vegetable intake on cancer prevention and tested its antimicrobial activity against *H. pylori*. An aqueous extract of garlic cloves was standardized for its thiosulfinate concentration and was tested for its antimicrobial activity on *H. pylori* grown on chocolate agar plates. MIC was determined at 40  $\mu\text{g}/\text{ml}$  of thiosulfinate. *S. aureus* tested under the same conditions was not susceptible to garlic extract up to the maximum thiosulfinate concentration tested (160  $\mu\text{g}/\text{ml}$ ). The authors suggested that the sensitivity of *H. pylori* to garlic extract at such low concentration may be related to the reported lower risk of stomach cancer in those with a high allium vegetable intake.

Using direct pre-infection incubation assays, Weber et al. (1992) reported the *in vitro* virucidal effects of fresh garlic extract, its polar fraction, and the following garlic-associated compounds: diallyl thiosulfinate (allicin), allyl methyl thiosulfinate, methyl allyl thiosulfinate, ajoene, alliin, deoxyalliin, diallyl disulfide, and diallyl trisulfide. Activity was determined against selected

viruses, including herpes simplex virus type I, herpes simplex virus type 2, parainfluenza virus type 3, vaccinia virus, vesicular stomatitis virus, and human rhinovirus type 2. The order for virucidal activity generally was ajoene > allicin > allyl methyl thiosulfinate > methyl allyl thiosulfinate. Ajoene was found in oil-macerates of garlic but not in fresh garlic extracts. No activity was found for the garlic polar fraction, alliin, deoxyalliin, diallyl disulfide, or diallyl trisulfide. Fresh garlic extract, in which thiosulfinates appeared to be the active components, was virucidal to each virus tested. The predominant thiosulfinate in fresh garlic extract was allicin. Lack of reduction in yields of infectious virus indicated undetectable levels of intracellular antiviral activity for either allicin or fresh garlic extract. Furthermore, concentrations that were virucidal were also toxic to HeLa and Vero cells. Virucidal assay results were not influenced by cytotoxicity because the compounds were diluted below toxic levels prior to assaying for infectious virus. These results indicate that virucidal activity and cytotoxicity may have depended upon the viral envelope and cell membrane, respectively. However, the authors concluded that the activity against non-enveloped virus may have been due to inhibition of viral adsorption or penetration.

Diallyl trisulfide, a chemically stable final transformation product of allicin, was synthesized in 1981 in China and was used for treatment of bacterial, fungal, and parasitic infections in humans. Lun et al. (1994) investigated the activity of diallyl trisulfide in several important protozoan parasites *in vitro*. The IC<sub>50</sub> (concentration that inhibits metabolism or growth of parasites by 50%) for *Trypanosoma brucei brucei*, *T.b. rhodesiense*, *T. b. gambiense*, *T. evansi*, *T. congolense*, and *T. equiperdum* was in the range of 0.8 to 5.5 µg/ml. IC<sub>50</sub> values were 59 µg/ml for *Entamoeba histolytica* and 14 µg/ml for *Giardia lamblia*. The cytotoxicity of the compound was evaluated on two fibroblast cell lines (MASEF, Mastomys natalensis embryo fibroblast and HEFL-12, human embryo fibroblast) *in vitro*. The maximum tolerated concentration for both cell lines was 25 µg/ml. These results indicated that diallyl trisulfide has potential to be used for treatment of several human and animal parasitic diseases. In a recent study, allicin was shown to inhibit the ability of *Entamoeba histolytica* trophozoites to destroy monolayers of baby hamster kidney cells (Ankri et al., 1997). Allicin has strongly inhibited cysteine proteinases, an important contributor to amebic virulence, as well as the alcohol dehydrogenase system of the parasite.

## AJOENE

Yoshida et al. (1987) reported the antifungal activity of six fractions derived from garlic in an *in vitro* system. Ajoene had the strongest activity in these fractions. The growth of both *Aspergillus niger* and *C. albicans* was inhibited by ajoene at less than 20 µg/ml.

Ajoene exhibits broad-spectrum antimicrobial activity (Naganawa et al., 1996). Growth of gram-positive bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, and *Streptomyces griseus*, was inhibited at 5 µg/ml of ajoene. *S. aureus* and *Lactobacillus plantarum* also were inhibited below 20 µg/ml of ajoene. For gram-negative bacteria, such as *E. coli*, *Klebsiella pneumoniae*, and *Xanthomonas maltophilia*, MICs were between 100 and 160 µg/ml. Ajoene also inhibited yeast growth at concentrations below 20 µg/ml. The microbicidal effect of ajoene on growing cells was observed at slightly higher concentrations than the corresponding MICs. *B. cereus* and *Saccharomyces cerevisiae* ( $10^5$  cfu/ml) were killed at 30 µg/ml of ajoene in 24 hours. However, the MIC for resting cells were at 10 to 100 times higher. The disulfide bond in ajoene appears to be necessary for the antimicrobial activity of ajoene, because reduction by cysteine, which reacts with disulfide bonds, abolished its antimicrobial activity.

Recently, Yoshida et al. (1998) isolated a compound showing antimicrobial activity from an oil-macerated garlic extract by silica gel column chromatography and preparative TLC. On the basis of the results of NMR and MS analyses, the compound was identified as Z-4,5,9-trithiadeca-1,6-diene-9-oxide (Z-10-devinylajoene; Z-10-DA). Z-10-DA exhibited a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria as well as yeasts. The antimicrobial activity of Z-10-DA was comparable to that of Z-ajoene, but was superior to that of E-ajoene. Z-10-DA and Z-ajoene are different in respect to substitution of the allyl group by the methyl group flanking a sulfinyl group. This result suggests that substitution by the methyl group would also be effective for the inhibition of microbial growth.

## MULTIFUNCTIONALITY

Garlic has attained a firm place in folk medicine for centuries. In addition to antimicrobial properties, garlic could elicit multifunctional effects to benefit human health. Garlic is capable of lowering blood cholesterol and reducing secondary vascular changes. It also raises fibrinolytic activity and inhibits thrombocyte aggregation. Therefore, garlic contains highly active therapeutic principles that appear to be particularly suitable for prophylaxis of arteriosclerosis (Ernest, 1981).

Allicin inhibits human platelet aggregation *in vitro* without affecting cyclooxygenase or thromboxane synthase activity or cyclic adenosine monophosphate levels (Mayeux et al., 1988). Allicin does not alter the activity of vascular prostacyclin synthase. However, it inhibits ionophore A23187-stimulated human neutrophil lysosomal enzyme release. *In vivo*, allicin dilates the mesenteric circulation independent of prostaglandin release or a beta-adrenergic mechanism.

Garlic has been touted as effective against diseases, in the pathophysiology of which reactive oxygen species (ROS) have been implicated. Effectiveness of garlic could be due to its ability to scavenge ROS. Prasad et al. (1995) investigated the ability of allicin contained in the commercial preparation "Garlicin" to scavenge hydroxyl radicals ( $\cdot\text{OH}$ ) using high pressure liquid chromatographic (HPLC) method. The  $\cdot\text{OH}$  radical was generated by photolysis of  $\text{H}_2\text{O}_2$  (1.25–10  $\mu\text{moles/ml}$ ) with ultraviolet light and was trapped with salicylic acid, which is hydroxylated to produce  $\cdot\text{OH}$  adduct products 2,3- and 2,5-dihydroxybenzoic acid (DHBA).  $\text{H}_2\text{O}_2$  produced a concentration-dependent  $\cdot\text{OH}$  as estimated by  $\cdot\text{OH}$  adduct products 2,3-DHBA and 2,5-DHBA. Allicin equivalent in "Garlicin" (1.8, 3.6, 7.2, 14.4, 21.6, 28.8, and 36  $\mu\text{g}$ ) produced concentration-dependent decreases in the formation of 2,3-DHBA and 2,5-DHBA. The inhibition of formation of 2,3-DHBA and 2,5-DHBA with 1.8  $\mu\text{g/ml}$  was 32.36% and 43.2%, respectively, while with 36.0  $\mu\text{g/ml}$ , the inhibition was approximately 94.0% and 90.0%, respectively. The decrease in  $\cdot\text{OH}$  adduct products was due to scavenging of  $\cdot\text{OH}$  and not by scavenging of formed  $\cdot\text{OH}$  adduct products. Allicin prevented the lipid peroxidation of liver homogenate in a concentration-dependent manner. These results suggest that allicin scavenges  $\cdot\text{OH}$  and that "Garlicin" has antioxidant activity.

Zheng et al. (1997) recently reported the inhibitory effects of allicin on proliferation of tumor cells. The effect was associated with the cell cycle blockage of S/G2M boundary phase and induction of apoptosis.

## PAM—POLYPHENOLS FROM TEA

Green tea is abundant with polyphenols, i.e., epigallocatechingallate [(-)-EGCg], epigallocatechin [(-)EGC], and epicatechingallate [(-)ECG]. These low molecular weight catechin derivatives could inhibit growth of cariogenic bacteria, *Streptococcus mutans*, and *Streptococcus sobrinus* in a dose-dependent manner. These cariogenic bacteria synthesize water-soluble and insoluble glucans that mediate bacterial cell adherence to tooth surface (Hamada and Slade, 1980). EGCg and ECG (25–30  $\mu\text{g/ml}$ ) completely inhibit glucan synthesis. This inhibitory effect is attributed to the ester-linked galloyl moiety of EGCg and ECG. *In vivo* experiments indicated that the dental caries score was distinctly lower in rats fed with confectioneries containing tea polyphenols (Nishihara et al., 1993). Also, chewing gum added with tea polyphenols was found effective in decreasing dental plaque formation in humans. Experiments of mouth rinsing with water containing green tea polyphenols resulted in significant reduction in dental plaque formation. A cup of green tea after lunch also resulted in reduction of dental caries risk in school children (Onisi, 1985).

Green tea polyphenols (catechins) also inhibit the collagenase activity, one of the virulent factors of periodontal disease (Makimura et al., 1993).

Administration of tea polyphenols through diet or drinking water reduced the occurrence of periodontal disease in mice challenged with *Actinomyces viscosus* (Kato, 1995). EGCg (250–500 µg/ml) strongly inhibited the growth of three strains of *Porphyromonas gingivalis*. Furthermore, EGCg at 125 µg/ml completely blocked the adherence of *Porphyromonas* species to eucaryotic cells.

Tea polyphenols also elicit antiviral effects against a variety of pathogens (Okubo and Juneja, 1997). Green (1949) reported inhibitory effects of black tea extract against proliferation of influenza A virus in embryonated eggs. Green tea leave extract also elicits antiviral activity against vaccina virus, herpes simplex virus, coxsackie virus B6, and polio virus 1 (John and Mukundan, 1979). EGCg from green tea and teafalvin digallate (TF3) from black tea could block the infectivity of both rotavirus and enterovirus in cultured rhesus monkey kidney MA 104 cells (Mukoyama et al., 1991) and influenza A and B virus in Madin-Darby canine kidney (MDCK) cells (Nakayama et al., 1993). EGCg and TF3 could also inhibit hemagglutination activity of influenza virus. Finally, tea polyphenols strongly inhibit the propagation of rotavirus cultured in rhesus monkey kidney MA 104 cells.

Nakane and Ono (1990) reported that the certain polyphenols and several other flavonoids from tea were strong inhibitors of reverse transcriptase of HIV (human immunodeficiency virus) and several DNA- and RNA-polymerases.

## MULTIFUNCTIONALITY

The prebiotic effect of tea polyphenols to induce the proliferation of beneficial intestinal microflora was suggested. Addition of methanol extract of green tea leaves (0.1%) induced a slight or moderate growth of *Bifidobacterium adolescentis*, *B. longum*, *B. breve*, *B. infantis*, *Lactobacillus casei*, and *L. salivarius*. Kakuda et al. (1991) observed an enhanced growth of *Bifidobacterium adolescentis* in the presence of water extracts of green tea marketed as "Gyokuro" and "Sencha." In contrast, *Clostridium perfringens*, *Bacteroides fragilis* and *Eubacterium lentum* failed to grow under similar conditions. The crude extracts of Gyokuro were more effective in enhancing growth of bifidobacteria than Sencha at an equivalent concentration. The authors suggested that the prebiotic effect of tea extract on the growth of bifidobacteria was due to the nutritive effects of the inorganic (potassium and phosphorus) and organic substances (several free amino acids and saccharides) contained in the extract.

EGCg, EGC, and ECG are oxidized when exposed to atmospheric oxygen. This property has been attributed in free radical scavenging ability of tea polyphenols. These compounds, therefore, are widely used as natural antioxidants to prevent oxidation of edible oils and to block discoloration of carotene-based foods (Koketsu, 1997). Tea polyphenols also demonstrate antioxidant

TABLE 1. Antimicrobial spectrum of phytoantimicrobial agents.

PAM Source	Susceptible Microorganism	Reference
Essential Oils		
Anise oil	<i>Lactobacillus curvatus/Saccharomyces cerevisiae</i>	Lachowicz et al. (1998)
Sweet linalool	<i>Pseudomonas</i> sp.	Wan et al. (1998)
Basil methyl chavicol	<i>Aeromonas hydrophila/Pseudomonas fluorescens</i>	Wan et al. (1998)
Bay and thyme oils	<i>Campylobacter jejuni</i>	Smith-Palmer et al. (1998)
Nutmeg oil	<i>Listeria monocytogenes</i>	Smith-Palmer et al. (1998)
Dill oil	<i>Lactobacillus buchneri/Saccharomyces vini</i>	Shcherbanovsky (1975)
<i>Achillea fragrantissima</i>	<i>Candida albicans</i>	Barel et al. (1991)
<i>Cedronella canariensis</i>	<i>Bordetella bronchiseptica/Cryptococcus albidus</i>	Lopez-Garcia et al. (1992)
<i>Hoslundia opposita</i>	<i>Aspergillus niger/Acinetobacter calcoacetalica/Bronchothrix thermosphaeta/Flavobacterium</i> sp.	Gundidza et al. (1992)
Camphor/camphene	<i>Escherichia coli/Aspergillus</i> sp./ <i>Candida albicans</i>	Tirillini et al. (1996)
	<i>Trichophyton mentagrophytes/Pseudomonas</i> sp.	
	<i>Staphylococcus aureus/Bacillus subtilis</i>	Al-Meshal (1986)
<i>Ducrosia ismaelis</i> Asch.		
Spices		
Cinnamon	<i>Bacillus subtilis</i>	Fabian et al. (1939)
	<i>Candida</i> sp./ <i>Kloeckera</i> sp./ <i>Rhodotorula</i> sp.	Conner and Beuchat (1984)
Cloves	<i>Staphylococcus aureus</i>	Fabian et al. (1939)
	<i>Candida albicans</i>	Briozzo et al. (1989)
Rosemary	<i>Salmonella typhimurium/S. aureus</i>	Farbood et al. (1976)
	<i>Clostridium botulinum</i>	Huhtanen (1980)
Mustard/black pepper	<i>Vibrio parahaemolyticus</i>	Beuchat (1976)
Turmeric	<i>Bacillus cereus/S. aureus/E. coli/L. plantarum</i>	Bhavani Shankar and Sreenivasa Murthy (1979)
<i>Aframomum danielli</i>	<i>Salmonella enteritidis/Pseudomonas</i> sp./ <i>S. aureus</i>	Adegoke and Skura (1994)
	<i>Aspergillus</i> sp./ <i>Proteus vulgaris/Streptococcus</i> sp.	
Fruit/Vegetables		
Carrots	<i>Listeria monocytogenes</i>	Beuchat et al. (1994)
	<i>Leuconostoc mesenteroides/E. coli/S. aureus/Pseudomonas</i> sp.	Babic et al. (1994)
	<i>Candida lambica</i>	
White potatoes	<i>Aspergillus parasiticus</i>	Swaminathan and Koehler (1976)

TABLE 1. (continued).

Cabbage	Enterobacteriaceae	Shofran et al. (1998)
Soybeans	<i>E. coli</i> / <i>S. aureus</i> / <i>Streptococcus pyogenes</i>	Ito et al. (1995)
Garlic	<i>B. subtilis</i> / <i>Serratia marcescens</i> / <i>Mycobacterium</i> sp.	Walton et al. (1936)
	<i>Pseudomonas aeruginosa</i> / <i>S. aureus</i>	Dankert et al. (1979)
	<i>Salmonella typhimurium</i>	Feldberg et al. (1988)
	<i>Shigella</i> sp./ <i>E. coli</i>	Chowdhury et al. (1991)
	<i>Helicobacter pylori</i>	Sivam et al. (1997)
	<i>Trypanosoma</i> sp./ <i>Entamoeba</i> sp./ <i>Giardia</i> sp.	Lun et al. (1994)
	<i>Klebsiella</i> sp./ <i>Xanthomonas maltophilia</i>	Naganawa et al. (1996)
Herbs		
Green tea	<i>Streptococcus mutans</i> / <i>Streptococcus sobrinus</i>	Harnada and Slade (1980)
	<i>Actinomyces viscosus</i> / <i>Porphyromonas</i> sp.	Katoh (1995)
	<i>Influenza virus</i> / <i>Vaccinia virus</i> / <i>Herpes Simplex</i> /	John and Mukundan (1979)
	<i>Coxasackie virus B6</i> / <i>Polio virus</i>	
	<i>Calliandra portoricensis</i> <i>E. coli</i> / <i>S. aureus</i> / <i>Streptococcus faecalis</i>	Aguwa and Lawal (1988)
<i>Yucca shidigera</i>	<i>Streptococcus bovis</i> / <i>Butyrivibrio fibrisolvens</i>	Wallace et al. (1994)
<i>Bridelia ferruginea</i>	<i>Staphylococcus</i> sp./ <i>Streptococcus</i> sp./ <i>E. coli</i> / <i>Proteus</i> sp./ <i>Klebsiella</i> sp./ <i>Candida albicans</i>	Irobi et al. (1994)
<i>Camillia sinensis</i>	<i>Microsporum audouinii</i>	Sagesaka et al. (1996)
<i>Arctotis auriculata</i>	<i>Mycobacterium smegmatis</i> / <i>Pseudomonas</i> sp.	Salie et al. (1996)
<i>Uvaria chamae</i>	<i>S. aureus</i> / <i>B. subtilis</i> / <i>Mycobacterium smegmatis</i>	Hufford and Lasswell (1978)
<i>Amaranthaceae</i>	<i>Mycobacterium phlei</i>	Pomilio et al. (1992)
<i>Tecoma stans</i>	<i>Candida albicans</i>	Binutu and Lajubutu (1994)
<i>Tagates minuta</i>	<i>Lactobacillus</i> sp./ <i>Zymomonas</i> sp.	Tereschuk et al. (1997)
<i>Ceanothus americanus</i>	<i>Streptococcus mutans</i> / <i>Antinomycetes viscosus</i> /	Li et al. (1997)
	<i>Porphyromonas gingivalis</i> / <i>Prevotella intermedia</i>	
<i>Prosopis juliflora</i>	<i>Candida</i> sp./ <i>Streptococcus</i> sp./ <i>Bacillus subtilis</i>	Aqueel et al. (1998)
	<i>Corynebacterium diphtheriae</i> / <i>Shigella</i> sp./ <i>Salmonella</i> sp./ <i>Vibrio</i> sp./ <i>Aeromonas</i> sp.	
<i>Ziziphus abyssinica</i>	<i>S. aureus</i> / <i>E. coli</i> / <i>C. albicans</i>	Gundidza and Sibanda (1991)
<i>A. hilotti</i>	<i>Clostridium perfringens</i> / <i>E. coli</i> / <i>Salmonella</i> sp.	Sotohy et al. (1995)
<i>Vicia faba</i>	<i>Saccharomyces cerevisiae</i> / <i>C. albicans</i>	Zhang and Lewis (1997)

activity *in vivo* as well as *in vitro* and could prevent oxidative impairment of cells.

EGCg has been suggested in reducing the risk of various tumors *in vivo*. EGCg could limit progression of duodenal carcinoma induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Fujita et al., 1989). In addition, green tea polyphenols have been shown to inhibit proliferation of intestinal clostridia *in vitro* and *in vivo* (Ahn et al., 1991). These organisms are associated in the biotransformation of various ingested or endogenously formed compounds into potential carcinogens such as *N*-nitroso compounds or aromatic steroids. The following inhibitory mechanisms, including nitration reactions, growth of intestinal clostridia, biochemical signals of tumor initiation, biochemical signals of tumor promotion, and antioxidative properties, could possibly contribute to the antitumor activity of green tea polyphenols.

## CONCLUSIONS

The antimicrobial spectrum of PAM compounds is summarized in Table 1. Recent consumer trends favoring consumption of natural foods have significant implications for the use of synthetic additives by food processors. Microbial resistance to synthetic antimicrobials such as with the vancomycin-resistant enterococci, emergence of new food-borne pathogens such as the *E. coli* O157:H7 and rapid rise of immunocompromised individuals in the consumer population are the critical factors instigating search for effective natural preservatives. An estimated annual increase of 4.1% use of traditional food preservatives is projected through year 2002 (Tollefson, 1995). PAM compounds with multifunctional benefits and proven safety and tolerance records are undoubtedly the most attractive food additives. However, adaptation of PAM compounds to modern food processing and innovative food microbial technology to optimize their multifunctional efficacy is warranted.

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# The Protective Effect of Tea on Cancer: Human Evidence

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## INTRODUCTION

TEA is the most widely consumed beverage in the world, and the history of tea drinking can be traced back to the ancient Shen-Nong time (2737 BC). In some parts of Asia (i.e., China, Japan, and India), tea is not only a beverage but also a part of traditional culture. Although tea is only produced in a relatively small number of countries (e.g., China, India, Japan, Sri-Lanka, and some North Africa countries), tea products are available almost anywhere in the world. Various types of tea are being manufactured from the leaves of the same tea plant, *Camellia sinensis*. Among the approximately 2.5 million metric tons of dried tea leaves manufactured annually, black tea accounts for about 78%; green tea, including jasmine tea, 20%; and oolong tea, 2% (Mukhtar et al., 1994; Stoner and Mukhtar, 1995). However, it should be pointed out that most herbal tea products in the Western markets actually contain no tea or very little tea.

The health effects of tea were first mentioned in the *Shen-Nong* (the first legendary herbal doctor in China) *Herbal*, in which it was stated that tea is effective in detoxifying 72 toxicants. Specific health effects of tea were further documented in the first Chinese pharmacopeia, *Classics of Materia Medica* (Li, Shizhen, 1578 AD). Among the health functions of tea, its possible protective effects on cancer have been studied and reported more often than other health effects.

The protective effects of tea on cancer were first shown in some *in vitro* and *in vivo* short-term mutagenicity tests, such as Ames test, etc., and then were confirmed in a few transplantable tumor models in mice as well as quite

a number of chemical carcinogenesis models in mice and rats. However, the epidemiological evidence of the preventive effects of tea on human cancers was not consistent, although a number of biologically plausible mechanisms on the protective effects of tea on cancer formation have been suggested. Whether tea drinking could be one of the recommendations in dietary guidelines for cancer prevention or, a step further, whether tea ingredients could be developed as chemopreventive agents for some subpopulations with high risk for cancer depend on further human evidence, especially on randomized, controlled intervention trials.

This chapter will focus on three clinical intervention trials recently conducted by our research group in different high-risk population groups in China using tea and tea ingredients. However, in order to put these studies in the context of a bigger picture, the chemistry of tea, laboratory studies, epidemiological studies, and mechanistic studies will also be reviewed briefly.

## THE CHEMISTRY OF TEA

The chemical composition of the tea leaf is very complex, and more than 400 chemical ingredients have been identified. Table 1 lists the major categories of tea ingredients. The content of each category of chemicals in tea leaves depends on the climate, soil, season, horticulture practices, and age of the leaf. Tea polyphenols, which account for 30 to 40% of the dried weight, are recognized as the major active ingredient, in respect to the health effects of tea. Because tea polyphenols are comprised of many individual components, the chemical composition of tea polyphenols varies with the manufacturing process and the type of tea.

Green tea is made by drying or steaming fresh tea (*Camellia sinensis*) leaves at high temperatures, and its chemical composition is similar to that of fresh leaves, which is characterized by its high content of tea polyphenols. Most of the tea polyphenols are flavanols, commonly known as catechins. The major green tea catechins are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin (EC),

TABLE 1. Composition of fresh tea leaves (%).

Polyphenols	36.0	Carbohydrates	25.0
Methylxanthines	3.5	Protein	15.0
Amino acids	4.0	Lignin	6.5
Organic acids	1.5	Lipids	2.0
Carotenoids	< 0.1	Chlorophyll, etc.	0.5
Volatiles	< 0.1	Ash	5.0

Source: Graham (1992).

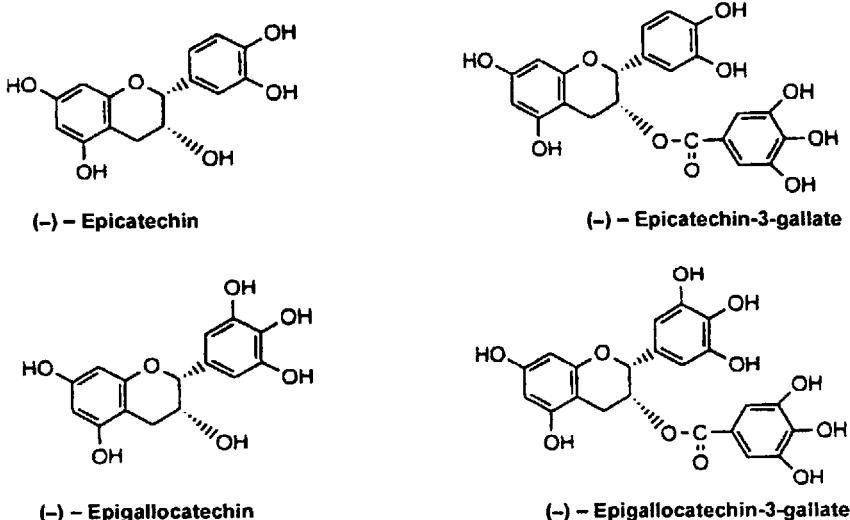
(+)-gallocatechin, and (+)-catechin (Figure 1). In addition, in the tea leaves, there is caffeine, theobromine, theophylline, phenolic acids, polysaccharides, etc., which only account for small proportions of the total composition.

During the process of black tea production, the tea catechins are oxidized (fermented) to theaflavins, thearubigens, and other oligomers (Figure 1). The term tea pigments is sometimes used to refer to the whole oxidized product of tea polyphenols, a mixture of color compounds mainly comprised of theaflavins and thearubigens. While theaflavins (1–2% of dry weight) determines the flavor and quality of black tea, thearubigens (10–20% of dry weight), often bound to peptides or proteins, is responsible for the dark color of black tea. In contrast to the tea catechins in the green tea, the components and relative proportion of individual components of theaflavins and thearubigens in black tea are less well characterized. The comparison of major polyphenolic components in green tea and black tea is presented in Table 2.

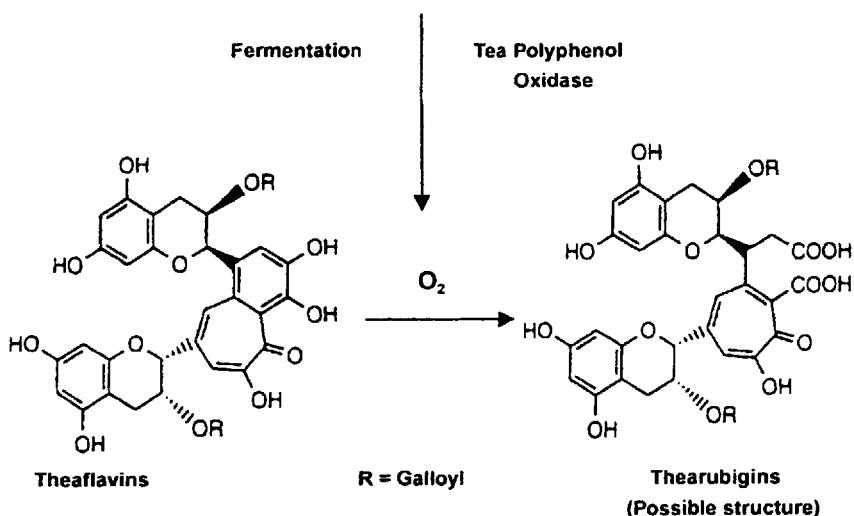
Oolong tea has been subjected to a shorter time of oxidation during processing, as compared with black tea. Therefore, it is also referred as half fermented tea, which contains considerable amounts of both tea polyphenols and theaflavins and thearubigens. The composition of polyphenolic components in oolong tea is between that of green tea and black tea.

## LABORATORY STUDIES ON THE PREVENTIVE EFFECTS OF TEA ON CELL MUTATION

Mutagenicity tests, which are often used to study the potential carcinogenicity of genotoxic carcinogens, have been conducted to study the possible anticarcinogenic effects of tea and tea ingredients in earlier studies and to screen the active components of tea in later studies. The *in vitro* and *in vivo* test systems used included Ames test (*Salmonella typhimurium*), *Escherichia Coli*, *Bacillus subtilis*, and BALB/C 3T3 cell transformation, as well as SCE, gene forward mutation, and micronuclei and chromosome aberration in V79 cells (Chen, 1992; NCI, 1996). The carcinogens used in these test systems included benz(a)pyrene, aflatoxin B1, 4-nitro-quinoline-N-oxide, nitrosomethylurea, 2-aminofluorene, 3-methylcholanthrene, mitomycin C, fluorouracil, mustargen, Me IQ, coal tar, fried fish extract, and cigarette smoke condensate (Chen, 1992; NCI, 1996). On the other hand, the samples of tea and tea components tested included water extracts of green, black, jasmine, and oolong tea, as well as green tea polyphenols (various purities), individual catechins (EGCG, EGC, ECG, EC), tea pigments, caffeine, polysaccharides, etc. (Chen, 1992; Han et al., 1997). The overall results indicated that all the tea and tea ingredients tested significantly inhibited the mutagenesis in all the test systems and with significant dose-response relationships. As an example, in our recent study on the screening of active ingredients of tea, a batch of short-term biological



### Major Components of Green Tea



### Major Components of Black Tea

**Figure 1** Chemical structure of major catechins, theaflavins, and thearubigens. Source: Yang and Wang (1993).

TABLE 2. Principal polyphenolic components in green and black tea (w/w % of extract solids).

Components	Green Tea	Black Tea
Catechins	30-42	3-10
Flavonols	5-10	6-8
Other flavonoids	2-4	-
Theogallin	2-3	-
Gallic acid	0.5	-
Quinic acid	2.0	-
Theanine	4-6	-
Methylxanthines	7-9	8-11
Theaflavins	-	3-6
Thearubigens	-	12-18

Source: Katiyar and Mukhtar (1996).

assays was used. Among the assays, V79 cell gene forward mutation and micronuclei formation tests were used to test the effects of tea on the initiation phase of carcinogenesis; a metabolic cooperation test was used to test the effects on the promotion phase; and Hela cell survival and growth was used to test the effects on the progression phase. In summary, the water extract of various tea (green, black, oolong, caffeinated, and decaffeinated), tea polyphenols (different purity), individual catechins (EGCG, EGC, ECG, EC), tea pigments, tea caffeine, and tea polysaccharides all showed certain protective effects in the initiation, promotion, and progression phases (Table 3). However, if the inhibitory potency is assessed on an equal concentration basis, the potency of tea polyphenols in the mutagenesis assays was usually not as strong as the whole water extract of green tea (Table 4). This implies that polyphenols are not the only active ingredient of tea and the combined effect of various components in the water extract is stronger than the individual effect. This finding is consistent with most findings in studies on herbal medicine, where only in rare cases, was an individual component of a herb eventually developed into a successful drug.

## LABORATORY STUDIES ON THE INHIBITION OF TUMORIGENESIS AND CARCINOGENESIS

The effects of tea and its ingredients on the inhibition of tumorigenesis and carcinogenesis have been extensively studied in chemical carcinogenesis models, mostly using mice or rats (Yang and Wang, 1993; NCI, 1996). The tea samples used included the water extract of various tea (green, black, jasmine, oolong, caffeinated, and decaffeinated), tea polyphenols, EGCG, and tea pigments. In most studies, the tea samples were given as drinking fluid,

TABLE 3. Effects of tea and tea ingredients in a batch of biological assays.

Assay	Carcinogen	Sample	Concentration (mg/ml)	Inhibition (%)
V79 cell gene forward mutation	MMC <sup>a</sup>	WEGT <sup>b</sup>	100	31.7
		Polyphenols	100	45.3
		Tea pigments	100	76.7
		EGCG	100	36.7
		ECG	50	31.7
		EGC	50	29.4
		EC	50	15.1
V79 cell micronuclei formation	MMC	WEGT	100	53.8
		Polyphenols	100	62.0
		Tea pigments	100	81.5
		EGCG	100	57.1
		ECG	50	79.0
		EGC	50	51.1
		EC	50	48.4
		Polysaccharide	100	39.9
		Caffeine	100	45.5
V79 cell metabolic cooperation	TPA	WEGT	50	12.3
		Polyphenols	50	29.0
		Tea pigments	20	49.3
		EGCG	20	31.2
		ECG	20	77.6
		EGC	20	30.1
		EC	20	28.5
		Polysaccharide	50	22.0
		Caffeine	50	33.4
HeLa cell growth in soft agar		WEGT	50	53.4
		Polyphenols	50	76.3
		Tea pigments	50	73.4
		EGCG	50	37.9
		ECG	50	65.2
		EGC	50	72.6
		EC	50	17.8
		Polysaccharide	50	46.8
		Caffeine	50	40.6

<sup>a</sup> MMC: mitomycin C.<sup>b</sup> TWEGT: water extract of green tea.

Source: Han et al. (1997); Liu et al. (1998).

except in the skin cancer model, in which topical treatment was used. The main target organs and the carcinogens used were skin, with 7, 12-dimethylbenz(a)anthracene (DMBA)/TPA, benz(a) pyrene (B(a)P), 3-methylcholanthraene (3-MC), and ultraviolet light; lung, with urethane, *N*-nitrosodiethylamine (NDEA), B(a)P, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); esophagus, with N-Nitrosomethylbenzylamine (NMBzA) and nitroso sarcosine;

TABLE 4. Comparison of antimutagenic potency between green tea water extract (WEGT) and green tea polyphenols (TP).

	WEGT	TP
Content (g, dry weight)	100	30
Concentration (mg/ml)	200	60
Inhibition rate <sup>a</sup> (%)	49.7	29.9

V79 cell gene forward mutation induced by mitomycin C.

Note: Based on the analytical data, there were 30% TP in the whole water extract of green tea (WEGT). Correspondingly, in a 200 mg/ml WEGT water solution, the TP concentration was 60 mg/ml. If TP is the only antimutagenic component in WEGT, the antimutagenic potency of 200 mg/ml WEGT and 60 mg/ml TP should be the same. However, the experiment result showed that 200 mg/ml WEGT was more potent than 60 mg/ml TP.

forestomach, with NMBzA, NDEA and B(a)P; duodenum and small intestine, with *N*-ethyl-*N'*-nitro*N*-nitroso-guanidine (ENNG); colon, with azoxymethane (AOM) and methylnitrosourea (MNU); liver, with aflatoxin B1 and NDEA; and mammary gland, with DMBA and PhIP. In summary, in all the animal models, all the tea samples tested showed inhibitory effects on tumorigeneis and/or carcinogenesis. The only exceptions were the studies reported by Wu et al. (1988) in which water extracts of oolong and jasmine tea did not inhibit mouse lung cancer induced by urethane and water extracts of oolong, jasmine, and green tea did not inhibit mouse skin cancer induced by B(a)P. The authors gave no explanations as to why these two experiments showed negative results, while their other experiments (MNNG-G.I. tumor and NDEA-lung tumor) (Wu et. al., 1988; Ruan et al., 1988) obtained positive results using the same tea samples.

In addition, tea preparations have been shown to cause partial regression of established skin papillomas in CD-1 mice induced chemically or by ultraviolet light; to suppress the growth of transplanted tumors (e.g., Ehrlich ascites carcinoma, hepatic carcinoma, and Sarcoma 180) in mice; and to prevent malignant tumor invasion and metastasis.

In conclusion, various teas and tea ingredients demonstrated unanimous protective effects on chemical carcinogeneis and transplantable tumors in experimental animals.

## SUGGESTED MECHANISMS FOR INHIBITION OF TUMORIGENESIS AND CARCINOGENESIS BY TEA PHYTOCHEMICALS

Several biologically plausible mechanisms for the inhibition of tumorigeneis and carcinogenesis in experimental animals by tea phytochemicals have been proposed (Chen, 1992; Yang and Wang, 1993; NCI, 1996). The main

hypotheses are antioxidative properties of tea, especially tea polyphenols; modulation of immune functions (Zhu et al., 1998); inhibition of nitrosation; inhibition of covalent binding between carcinogen and DNA; modulation of carcinogen metabolism; inhibition of oncogene expression; protection of the inhibition of intercellular communication by promoters; and inhibition of cell proliferation. Because the cancer formation is a very complex process with multiple phases it is very likely that the protective effects of tea and its ingredients involve several mechanisms and, during the intervention process, these mechanisms are interrelated (e.g., the reduction of DNA adduct formation and facilitation of carcinogen detoxification) and complimentary (the scavenging of free radicals and modulation of immune functions). Considering the differences in cancer process between man and animal, further studies on the mechanisms of tea should be incorporated into human clinical intervention trials.

## EPIDEMIOLOGICAL EVIDENCE

Although a large body of laboratory research data consistently showed that tea and its main ingredients have significant protective effects on tumorigenesis or carcinogenesis, the final conclusion has to be based on human evidence. Because tea is only a minor part of the complicated human lifestyle, it is extremely difficult to control all the confounding factors (e.g., diet, smoking, alcohol drinking, etc.) and find out the real effects of tea drinking on cancer incidence or mortality in any type of epidemiological studies.

Several authors have reviewed the epidemiologic literature on tea and cancer prevention (Blot et al., 1996; Fujiki et al., 1996; Katiyar and Mukhtar, 1996; NCI, 1996; Kohlmeier et al., 1997; Bushman, 1998). For practical reasons, most of the published data were case-controlled studies, and information on the frequency and amount of tea consumption was collected after the subject had developed cancer. In the first five reviews, the effects of both green tea and black tea were evaluated, while in the review by Bushman (1998), only information on green tea was collected. The overall message from these reviews is that, although a number of studies found a protective effect of tea drinking in several cancer sites, significant numbers of studies did not find that tea drinking was protective or it was found that tea drinking even increased cancer incidence. Although the high temperature of tea (scalding hot tea) and not tea per se was found to be associated with esophageal cancer, it could not explain the positive association for other cancer sites. In other words, in contrast to the strong and consistent evidence seen in the laboratory studies, current epidemiological studies did not show a consistent protective effect of tea drinking in real life. Some summary data from the review by Bushman (1998) on green tea are presented in Table 5. This conclusion on the epidemi-

TABLE 5. Green tea and cancer: epidemiological studies.

Country	Type of Study	Association	Authors
<b>Pancreatic Cancer</b>			
Japan	Case-control	Inverse	Goto et al. (1990)
Japan	Case-control	Positive	Mizuno et al. (1992)
China	Case-control	Inverse	Ji et al. (1997)
<b>Colorectal Cancer</b>			
China	Case-control	Inverse	Ji et al. (1997)
Japan	Case-control	Inverse	Kato et al. (1990)
Japan	Case-control	Inverse	Kono et al. (1991)
Japan	Case-control	Inverse	Tajima et al. (1985)
Japan	Case-control	Positive	Watanabe et al. (1984)
<b>Lung Cancer</b>			
Hong Kong	Case-control	Positive	Tewes et al. (1990)
China			
Japan	Case-control	Inverse	Ohno et al. (1985)

Source: Bushman (1998).

logic studies on tea and cancer was confirmed by a presentation by Dr. W.H. Chow, U.S. National Cancer Institute at the *Second International Symposium on Tea and Health*, September 1998, Washington, D.C.

## CLINICAL INTERVENTION TRIALS

It is generally agreed that intervention trials remain the most reliable approach to answer the question of whether tea has a protective effect in human cancer development. However, because cancer formation is a long-term process and the incidence of each individual cancer site is relatively low, a large sample population and long-term follow-up are necessary, which actually prohibits the conduction of such trials. And logically, it is very difficult to maintain a non-tea consumption control group that is comparable to the intervention group in other lifestyle aspects for a long time period. Therefore, we have chosen three high-risk population groups and applied multiple intermediate endpoints to evaluate the effects of tea and its ingredients on cancer prevention.

## ORAL LEUKOPLAKIA

Oral leukoplakia (Li et al., 1999) is a well-established precancerous lesion of oral cancer. In general, 2 to 12% of patients with oral leukoplakia will eventually develop malignant oral cancer, and in certain pathology types, the proportion could be as high as 15 to 40%.

Sixty-four cases of oral leukoplakia (36 men and 23 women) diagnosed by oral pathology were chosen from the Beijing Dental Hospital (Dr. Zeng Sun as collaborator). They were randomly divided equally into a tea-treated group and a control group. Patients in the treated group were given 3 gm of mixed tea in capsules (q.i.d.), and the lesions were painted topically with 10% mixed tea in glycerin. The mixed tea was provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences and was comprised of a dried mixture of the whole water extract of green tea (Long Jin), green tea polyphenols (40% purity), and tea pigments in the ratio of 4:1:1. Patients in the control group were given placebo capsules and were painted with glycerin. Twenty-nine subjects in the tea-treated group and 30 subjects in the control group completed the six-month trial.

After six months of tea intervention, partial regression of the oral lesions was observed in 11 of the 29 (37.9%) cases, no change was observed in 17 (58.6%) cases, and deterioration was observed in one (3.4%) case. In the control group, partial regression was found in three of the 30 (10.0%) cases, no change in 25 (83.3%) cases, and deterioration in two (6.7%) cases. The partial regression rate in the tea-treated group was significantly higher than that on the control group ( $p < 0.05$ ).

The frequency of micronucleated exfoliated buccal mucosa cells and the frequency of micronucleated cells and chromosome aberration in the peripheral blood lymphocytes were examined as biomarkers of DNA damage, which is a crucial mechanism in cancer process and a marker of early-carcinogenesis. The data in Table 6 show that frequency of micronucleated buccal cells in both lesion sites and normal sites were higher in the leukoplakia patients than in the normal subjects ( $p < 0.01$ ). In the same leukoplakia patients, the frequency of micronucleated cells was higher in the mucosa cells from the lesion sites than from the normal sites. After three and six-months of tea treatment,

TABLE 6. Frequency of micronucleated exfoliated buccal cells in leukoplakia patients (per 1,000 cells).

	Tea-Treated (n = 29)		Placebo Controls (n = 30)	
	Lesion	Normal Mucosa	Lesion	Normal Mucosa
Baseline	10.50 ± 5.29 <sup>a,b</sup>	5.20 ± 2.79 <sup>a</sup>	10.10 ± 4.07 <sup>a,b</sup>	5.12 ± 2.04 <sup>a</sup>
3-month	6.68 ± 3.21 <sup>c,d</sup>	3.89 ± 1.86 <sup>b</sup>	10.35 ± 4.07	4.82 ± 2.53
6-month	5.39 ± 3.05 <sup>c,d</sup>	3.05 ± 1.62 <sup>c,d</sup>	11.30 ± 4.29	5.46 ± 2.90

<sup>a</sup> $p < 0.01$ , compared with healthy controls by Poisson test.

<sup>b</sup> $p < 0.01$ , compared with normal mucosa by Poisson test.

<sup>c</sup> $p < 0.01$ , compared with baseline by Poisson test.

<sup>d</sup> $p < 0.01$ , compared with placebo controls by Poisson test.

<sup>e</sup> $p < 0.05$ , compared with baseline by Poisson test.

Source: Li et al. (1999). All values are mean ± SD; normal subjects (n = 20) 1.4 ± 0.6.

TABLE 7. The number of AgNOR dots per nucleus and PCNA index in leukoplakia lesions of oral mucosa before and after trial.

	Tea-Treated (n = 22)	Placebo Controls (n = 21)
AgNOR		
Baseline	6.34 ± 2.19	6.24 ± 2.01
6-month	4.44 ± 3.80 <sup>a,b</sup>	6.10 ± 2.71
PCNA index		
Baseline	36.2 ± 22.9	36.2 ± 22.9
6-month	24.3 ± 22.9	36.2 ± 22.9
6-month	24.3 ± 16.5 <sup>c,d</sup>	39.0 ± 23.4

<sup>a</sup>p < 0.01, compared with baseline by t-test.

<sup>b</sup>p < 0.05, compared with placebo controls by t-test.

<sup>c</sup>p < 0.05, compared with baseline by t-test.

<sup>d</sup>p < 0.05, compared with placebo controls by t-test.

Source: Li et al. (1999). All values are mean ± SD.

micronuclei formation in the cells from both lesion sites and normal sites decreased significantly ( $p < 0.01$ ) in the tea-treated group, but there was no change in the control group. The frequency of micronuclei and chromosome aberration in peripheral blood lymphocytes was also significantly reduced in the leukoplakia cases after treatment by the mixed tea for six months.

The biomarkers of cell proliferation (another important mechanism during carcinogenesis) measured using biopsy oral mucosa tissue included AgNOR (silver-stained nuclear organizer regions), PCNA (proliferation cell nuclear antigen), and EGFR (epidermal growth factor receptor) expression. After six months treatment, the number and volume of AGNOR dots and the PCNA index decreased significantly ( $p < 0.01$ ) in the tea-treated group, while no significant changes were found in the control group (Table 7). The percentage of EGFR-positive cells was reduced; however, it was not statistically significant due to the wide individual variation (Table 8).

The above results indicate that mixed tea treatment not only improved the clinical manifestations of precancerous oral lesions, but also protected against

TABLE 8. Percentage of EGFR positive cells in oral leukoplakia lesions before and after trial.

	Tea-Treated (n = 22)	Placebo Controls (n = 21)
Baseline	36.4 ± 25.8	35.8 ± 26.5
6-month	32.2 ± 20.4 <sup>a</sup>	36.7 ± 26.5

<sup>a</sup>Comparison of values between baseline and 6 months in tea-treated group, as well as between tea-treated group and placebo group, are not statistically significant by t-test,  $p > 0.05$ .

Source: Li et al. (1999). All values are mean ± SD.

DNA damage and inhibited cell proliferation of oral mucosa cells. This is in line with our animal studies, which showed a strong protective effect of the mixed tea on DMBA-induced oral tumors in golden Syrian hamsters by reducing tumor formation at buccal pouch, preventing DNA damage, and inhibiting cell proliferation (Li et al., 1999). Although there are limitations in the sample size and duration of intervention, the results from this trial have provided some direct evidence of the preventive effects of tea on human cancer.

## HABITUAL CIGARETTE SMOKERS

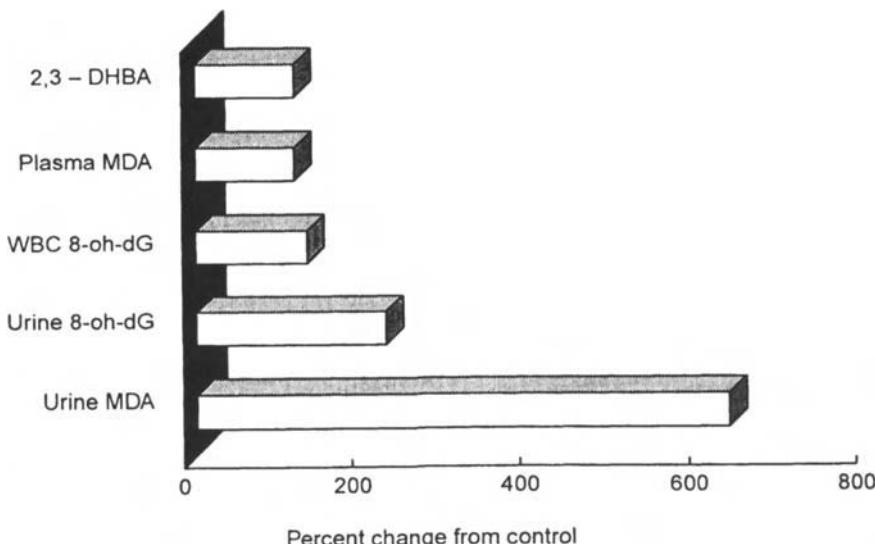
Cigarette smoking is an established cause of human cancer, as well as cardiovascular diseases. It has been demonstrated that cigarette smoking induces reactive oxygen species (Church and Pryor, 1985) and oxidative DNA damage (Piperakis et al., 1998; Howard et al., 1998). Therefore, oxidative stress may play an important role in the pathological changes seen with chronic smoking. Based on this hypothesis, a collaborative study of the effect of tea consumption on smoking-induced oxidative stress was carried out among our group (Dr. J. Klaunig's group at the Indiana University School of Medicine and Dr. C. S. Yang at the Rutgers University, U.S.)

Chinese male habitual cigarette smokers between the ages of 18 and 24 with similar diets and physical activity were given various types of tea, and a variety of oxidative stress biomarkers in blood and urine were measured. In the green tea (Long Jin) group, 20 subjects drank two cups of tea (3 g extracted in 150 ml hot water for 30 minutes, twice) and smoked two cigarettes one hour after tea drinking. In the control group, 20 subjects drank the same amount of hot water without tea and smoked the same amount of cigarettes. Another group, comprised of 20 non-smoking subjects, only drank hot water during the trial. The following end points of oxidative stress and DNA damage were measured after one and seven days of tea drinking and cigarette smoking: plasma and urine malondaldehyde (MDA); WBC and urine 8-hydroxy-2'-deoxyguanosine (8-oh-dG); urine 2,3-dihydroxyl benzoic acid (2,3-DHBA) (an aspirin metabolite indicating the amount of reactive oxygen radicals formed after consumption of 1 g of aspirin); micronucleated cells in oral mucosa; and micronucleated cells and chromosome aberration in peripheral blood lymphocytes. In addition, two other groups drank black tea (Lipton, New Jersey) and mixed tea (see above section on oral leukoplakia trial), respectively, and the same endpoints were measured. However, only the results from green tea drinking will be presented here, in conjunction with the results from another trial conducted in Indianapolis (Klaunig et al., 1999). The latter study was conducted in 27 men and women between the ages of 25 and 45 (12 smokers and 15 nonsmokers). Subjects in both groups 1 (smokers) and 2 (nonsmokers) received either green tea (2.75% in water) or a placebo with meals. Diet and physical activity were not controlled. Smoking behavior in the smoking sub-

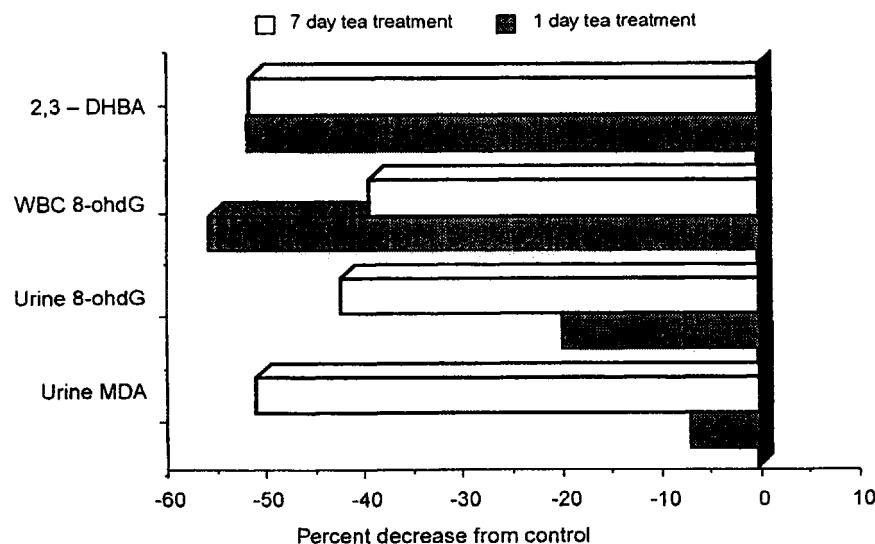
jects was allowed to continue as usual. The protocol involved no tea drinking for the first week followed by the drinking of tea or placebo for one week, followed by a washout period (no tea or placebo drinking), followed by one week of tea (or placebo) consumption. Blood and urine were collected after one week (no treatment), two weeks, three weeks, and four weeks of study for measurements of the same biomarkers.

The effect of smoking (without tea) on the oxidative stress endpoints measured in the two studies (combined) is shown in Figure 2. Oxidative stress parameters measured in blood and urine showed an increase in smokers compared with nonsmokers immediately (one hour) after smoking. White blood cell 8-oh-dG was increased in smokers to 1.7 fold of nonsmokers. Similarly, urine 8-oh-dG was approximately 2.3 fold greater in smokers than in nonsmokers. Urine (6.4 fold) and plasma (1.5 fold) lipid peroxidation were also significantly increased in smokers. The amount of reactive oxygen radicals formed (as measured by 2,3 DHBA formation in urine) was 1.5 fold greater in smokers. In general, smokers in China and American subjects exhibited similar increases (compared to their nonsmoker counterparts) in oxidative stress endpoints measured.

On the other hand, tea drinking showed significant protective effects on most of the above oxidative stress biomarkers in the two trials. In the China study, consumption of green tea (Figure 3) for one or seven days in smokers resulted in a significant decrease in most of the measurements from that seen



**Figure 2** Relative change in oxidative stress endpoints in smokers compared to non-smokers.  
Source: Klaunig et al. (1999).

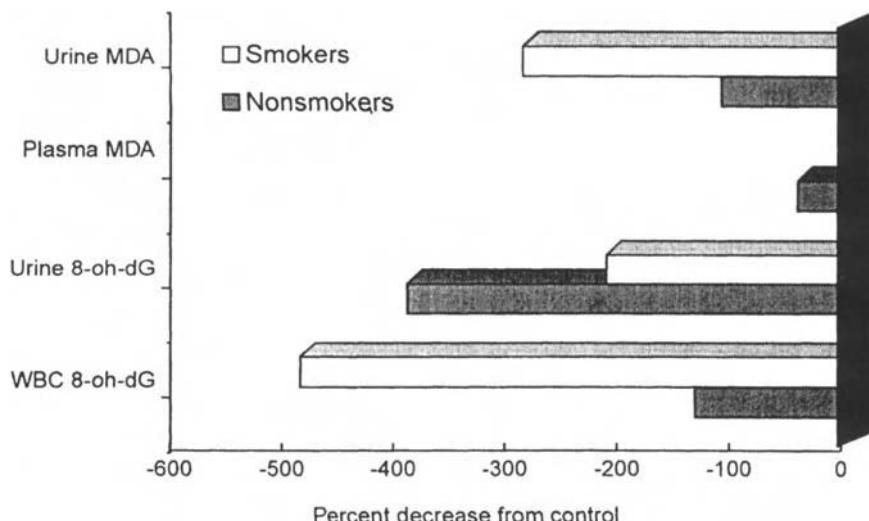


**Figure 3** Effect of green tea drinking on oxidative stress in Chinese smokers. Source: Klaunig et al. (1999).

at the 0 sampling time (Figure 2). Only plasma MDA showed no change from 0 time measurements. One day of tea treatment appeared to exert its greatest effect on WBC 8-oh-dG levels, while treatment with tea for seven days showed a greater effect on urinary oxidative stress measurements. The reduction of oxygen radical formation (2,3 DHBA) was equally decreased after either one or seven days of tea consumption. In the U.S. study, both smokers and nonsmokers exhibited a decrease in oxidative stress endpoints (as measured by mean percentage decrease from placebo treatment) following green tea consumption (Figure 4). Similar to that observed in the China study, plasma MDA was not decreased in smokers following green tea consumption.

The antioxidant properties have been shown in a number of animal and *in vitro* models. This is the first time that multiple biomarkers were used to investigate the antioxidant effects of tea in humans. The results from both the China study and the U.S. study showed that consumption of green tea in usual amounts reduced oxidative damage in smokers. Furthermore, our results from the DNA damage biomarkers showed that green tea consumption also decreased the frequency of micronucleated cells in oral mucosa, as well as chromosome aberration in peripheral blood lymphocytes (Table 9).

In conclusion, the above results showed that green tea functions as an antioxidant in humans. The antioxidant property seen in cigarette smokers provides indirect evidence suggesting that tea drinking may have protective effects against those tobacco-related cancers.



**Figure 4** Effect of green tea drinking on oxidative stress in American smokers. Source: Klaunig et al. (1999).

### ALPHA-FETOPROTEIN-POSITIVE SUBJECTS

Serum alpha-fetoprotein is a widely used marker for primary liver cancer detection. It was reported that in subjects with repeated low titer AFP positivity, the chance of developing primary liver cancer is extremely high (up to 5%). Therefore, in collaboration with Dr. Y. Xu at the Nanjing Medical University, we conducted a double-blind intervention trial in subjects in a high-risk area for liver cancer who met the following criteria: male, older than 30 years, hepatitis B virus surface antigen (HBsAG) positive, repeated AFP positive (titer 1:5–1:200), and with no manifestation of liver cancer. The selected subjects were randomly divided into a tea-treated group (80

**TABLE 9.** Effects of green tea drinking on frequency of micronucleated cells (per 1,000 cells) in oral mucosa and chromosome aberration in peripheral blood lymphocytes (per 100 cells) in smokers.

	Micronucleated Cells		Chromosome Aberration	
	Before	After	Before	After
Smoking control	5.2 ± 1.9	5.6 ± 2.1	1.9 ± 1.9	2.0 ± 2.0
Green tea drinking	5.2 ± 2.3	4.5 ± 1.8 <sup>a</sup>	1.9 ± 1.3	1.4 ± 1.4 <sup>a</sup>
Nonsmoking control	0.9 ± 0.9 <sup>b</sup>	—	0.3 ± 0.7 <sup>b</sup>	—

<sup>a</sup>p > 0.05, compared with values before trial as well as values of smoking controls.

<sup>b</sup>p < 0.01, compared with the green tea drinking group and the smoking control group.

subjects) given 2.4 g of mixed tea in capsules (see above section on oral leukoplakia) and a control group (78 subjects) who received placebo capsules.

During the 10-month intervention, three check-ups were carried out at three-month intervals, which include beta-ultrasound examination and blood assays. Thirty subjects (19%) were lost due to moving, other diseases, or relinquished cooperation. Early primary liver cancer was diagnosed in both groups: 12 cases (15%) in the tea-treated group and 11 cases (14%) in the control group. Serum biochemical markers measured included AFP, activity of transaminase (ALT), alkaline phosphatase (AKP), and gamma-glutamyl transpeptidase (gamma-GGT), as well as hepatitis B markers (HBsAG, anti-HB, HBeAg, anti-HBe, and anti-HBc). All the biochemical measurements showed no significant differences between before and after interventions, and between the tea-treated and the control groups.

In conclusion, the above results clearly showed that mixed tea treatment has no protective effect on liver cancer development in repeated low-titer AFP-positive patients. One possible explanation for this negative result is that these subjects were in quite advanced stages of liver cancer development, although the beta-ultrasound examination failed to detect any liver cancers at the baseline examination. This argument is supported by the extremely high incidence of liver cancer in these subjects within the 10 months. The other possible explanation is that tea is not able to protect against cancers of primarily biological etiology and mainly protects against cancers of primarily chemical etiology.

## CONCLUSIONS

While the anticarcinogenic effects of tea in animal models have been consistently reported by various authors, human epidemiologic studies examining tea consumption and cancer risk have produced equivocal results. This may be due to the fact that cancer is a disease with multiple etiological factors, and in traditional epidemiologic studies, it is hardly possible to control so many confounding lifestyle factors, including smoking, drinking, etc. Therefore, clinical intervention trial in high-risk populations is considered the best approach to find out whether tea is protective against human cancer.

Three clinical intervention trials studying the protective effects of tea on cancer in high-risk populations were conducted by our group using intermediate endpoints. Among the three trials, the study on oral leukoplakia (a precancerous lesion), using the mixed tea preparation, provided some direct evidence on oral cancer prevention, as well as evidence on the improvement of DNA damage and inhibition of cell proliferation. The second study in habitual

cigarette smokers on the effects on oxidative stress using multiple biomarkers showed antioxidant effects of tea in humans, and the results provided indirect evidence of the effects on the prevention of lung cancer and other tobacco-related cancers. Although the third study in repeated low-titer AFP positive subjects did not show any positive results on liver cancer prevention, the overall results of these intervention trials did show that tea drinking may have some promising effects in human cancer prevention. In addition, the results also showed that surrogate biomarkers could serve as intermediate endpoints in well-controlled randomized intervention trials. This has important complications, because intervention studies using cancer incidence as an endpoint usually need large sample size and long duration and are very difficult to have parallel control groups.

However, it should be pointed out that, in our oral leukoplakia trial, the number of subjects was not large enough and the intervention period was not long enough; and in the habitual cigarette smoker trial, the number of subjects in each group was also not large enough, the results of some of the biomarkers had quite large individual variation, and the number of cigarettes consumed was not large enough to produce stronger oxidative stress. Therefore, these trials need to be repeated in order to obtain convincing results on the protective effects of tea on cancer.

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# Effect of Genistein on Growth of Human Breast Cancer Cells *in vitro* and *in vivo*

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## INTRODUCTION

BREAST cancer is strongly associated with affluence, and occurrence rates can vary by as much as five- to 10-fold between countries. Asian women have a much lower incidence of breast cancer compared to those in Western countries. When these women migrate from Asian countries to Western countries, their incidence of breast cancer increases; and by the second generation, breast cancer risk is similar to those in the high-risk countries. These results strongly suggest that environmental factors, including diet, play a role in the etiology of breast cancer (Wynde, 1980; Willet, 1989). In general, Asian women consume diets low in fat and high in fruits and vegetables. Additionally, these women consume soy protein as a dietary staple. In recent years, soy has been the focus of considerable research for potential health benefits. These studies have focused on reduction of various chronic diseases; in fact, a health claim regarding soy and cardiovascular disease is in the final stages of approval by the Food and Drug Administration (FDA). Our research focus is on the soy phytoestrogen, genistein, and its possible growth-altering effects on estrogen-dependent breast cancers.

Several studies have been conducted to evaluate the potential protective effects of dietary soy on chemically induced mammary cancer in the rat. In a study conducted by Hawrylecicz et al. (1991) using chemically induced mammary cancer, rats fed soy protein diets had fewer tumors per rat as well as a significant reduction in total tumor weight. There was no change in food intake throughout the study. This study, as well as others (Troll et al., 1980), suggests that compounds in soy are chemopreventative. There are numerous

compounds present in soy that can act as chemoprevention agents (Messina et al., 1994). These include protease inhibitors, saponins, phytates, fiber, phytosterols, and isoflavones. There are three isoflavones present in soy: daidzein, genistein, and glycitein. These exist in nature as the glycoside. It is believed that the glycoside is cleaved into the aglucone by bacteria present in the lower intestine prior to absorption. Genistein is the most widely studied of the soy isoflavones; in fact, during the past decade, approximately 1000 articles have been published on genistein. Genistein has numerous biological activities (Akiyama et al., 1987; Farmakalikis et al., 1985; Miksicek, 1993; Setchell and Cassidy, 1999). We will focus on the anti-proliferative and estrogenic effects of genistein on growth of human breast cancer cells *in vitro* and *in vivo*. Genistein is expensive; prior to initiating the studies discussed in this manuscript, we (Chang et al., 1994) produced approximately 75 grams of genistein from organic precursors for use in long-term dietary studies.

## ANTI-PROLIFERATIVE EFFECTS OF GENISTEIN

Genistein, at concentrations greater than 20  $\mu\text{M}$ , inhibits cell proliferation of both estrogen-responsive (MCF-7) and estrogen-independent (MDA-468) human breast cancer cells *in vitro* (Peterson and Barnes, 1991; Monti and Sinha, 1994). Additionally, genistein at concentrations of 100  $\mu\text{M}$  has been shown to block tyrosine phosphorylation induced by 10  $\mu\text{M}$  of insulin (Pagliacci et al., 1994). These researchers and others (Matsukawa et al., 1993) have demonstrated that genistein blocked the cell cycle at G2/M at concentrations above 20  $\mu\text{M}$ . The block in cell cycle progression may be due to the known anti-tyrosine kinase activity of genistein. Tyrosine phosphorylation is associated with activation of cellular receptors involved in growth regulation and control of cell cycle in a variety of cell types.

We have conducted studies using estrogen-independent MDA 231 cells and have demonstrated that genistein will block growth of these human breast cancer cells in a dose-dependent manner at concentrations from 20 to 80  $\mu\text{M}$ . We followed these studies with cell cycle analysis and have demonstrated that genistein will block the cell cycle at G2/M at concentrations above 40  $\mu\text{M}$ . This effect was sustained for 72 hours. These cell culture studies with estrogen-independent human breast cancer cells are consistent with results obtained from other researchers using a variety of transformed human cancer cell types. We followed these studies with *in vivo* studies using the athymic mouse implanted with the estrogen-independent human breast cancer cells (Santell et al., 1998). To evaluate whether the *in vitro* effects observed could be reproduced *in vivo*, MDA-231 cells were implanted into several subcutaneous sites in athymic mice. Five weeks later, tumor size was measured, and the animals were sorted into two treatment groups, each group containing

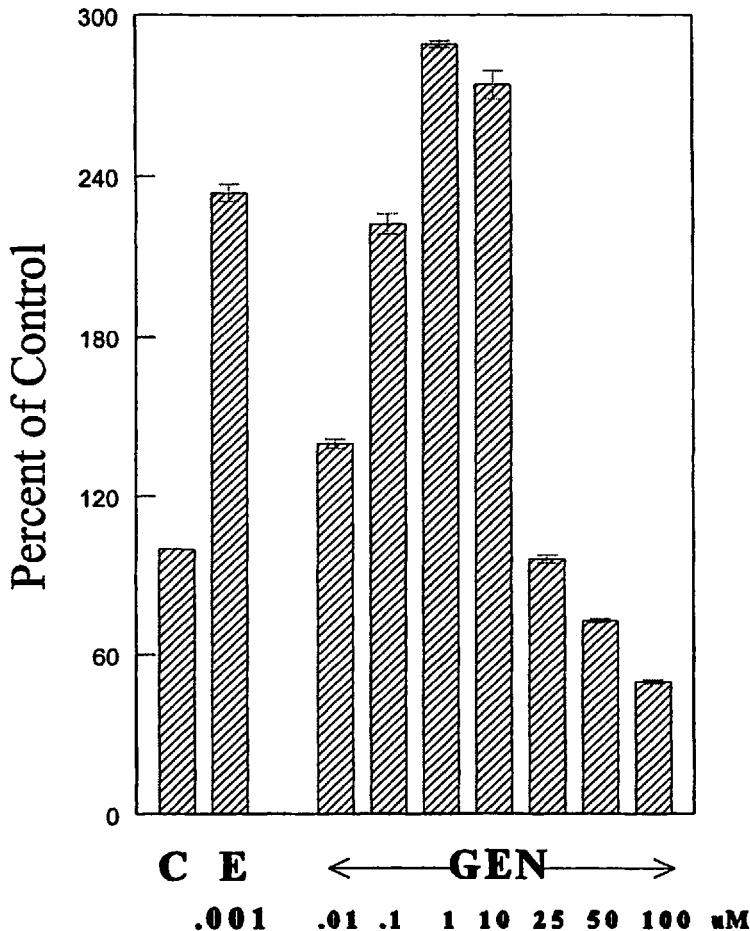
equal mean tumor size. Mice were administered genistein at 0 and 750 ppm in the AIN-93G diet and were fed *ad libitum* for five weeks. After five weeks, the mice were killed, tumors size was determined, and the mammary gland and uterus were removed for analysis. Change in tumor size was not significantly different in the 750 ppm genistein-treated mice compared to the AIN-93G control mice (Santell et al., 1998). In summary, genistein has an anti-proliferative effect on cultured estrogen-independent human breast cancer cells. It may not be possible to achieve concentrations above 20  $\mu\text{M}$  in the blood from dietary exposure of genistein. Because genistein plasma levels in humans consuming soy are 1  $\mu\text{M}$  or less, it is unlikely that dietary consumption of genistein (whether from soy or a supplement) will produce plasma levels of the free genistein near 20  $\mu\text{M}$ . Thus, the anti-proliferative effects observed *in vitro* may be difficult to achieve *in vivo*.

## ESTROGENIC ACTIVITY OF GENISTEIN

Estrogen and estrogen agonists act by initially binding to the estrogen receptor (ER). Once the ligand binds to the ER the ER undergoes transformation in which the chaperone protein (heat shock protein) dissociates and the DNA binding domain of the ER is exposed. Additionally, the bound ER forms a homodimer, and this dimer binds to estrogen responsive enhancers (ERE) upstream of estrogen-responsive genes (Kumar et al., 1986, 1987). Binding to the ERE initiates transcription of estrogen-responsive genes (Webster et al., 1988). These responsive genes are responsible for estrogen responses such as increases in uterine weight and in mammary gland proliferation and differentiation. Additionally, these responsive genes are responsible for the stimulation of growth of estrogen-dependent human breast cancer cells.

We conducted competitive-binding experiments with rat uterine cytosol and determined that genistein binds to the ER with an affinity 1/50 to 1/100 that of estradiol (Santell et al., 1997). Binding to ER suggests that genistein can produce an estrogenic response. One indicator that genistein can act as an estrogen agonist is to determine whether genistein can stimulate estrogen-dependent proliferation in estrogen-dependent human breast cancer (MCF-7) cells. In order to evaluate whether genistein will enhance estrogen-dependent (MCF-7) proliferation, we conducted a cell proliferation dose response study. MCF-7 cells were monitored in response to estradiol (1 nM) and various concentrations of genistein ranging from 0.01  $\mu\text{M}$  to 100  $\mu\text{M}$  (Figure 1). Data are expressed as percentage of the control cell cultures. These levels were chosen because genistein blood levels reported in animals and humans consuming diets high in genistein (such as soy-containing diets) have blood concentrations ranging from 0.1 to 6  $\mu\text{M}$  (Xu et al., 1994, 1995). Estradiol (1 nM) stimulated cell proliferation 2.4-fold over the control MCF-7 cells. Genistein

# MCF-7 Cell Proliferation



**Figure 1** Effects of estradiol and genistein on the growth of estrogenic responsive MCF-7 cells. MCF-7 cells were cultured in the presence of various concentrations of genistein (10 nM–100  $\mu$ M) for 96 hours, in IMEM media containing 5% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Proliferation was assessed by DNA content as measured using HOECHST reagent and fluorometric analysis. Fluorescence was measured by excitation at 350 nm and emission at 455 nm and was used to determine DNA content. The results (mean,  $n = 8$ ) are expressed relative to cells grown without genistein. C represents vehicle control, and E represents treatment with 1 nM of estradiol in the media.

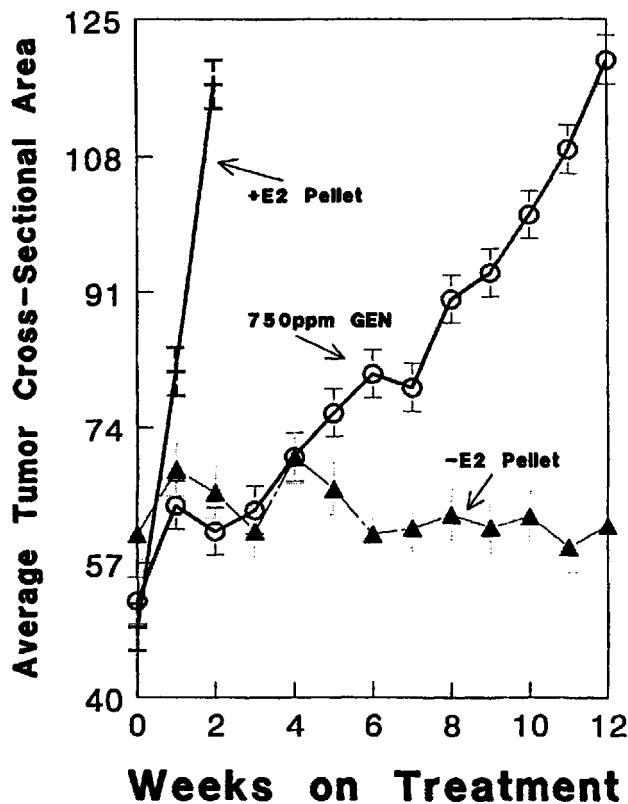
increased cell growth in a dose-dependent manner in the range of 0.01  $\mu\text{M}$  to 1  $\mu\text{M}$ . Maximal growth stimulation (approximately three-fold over control) was observed at 1  $\mu\text{M}$  and was sustained at this level of stimulation dose up to 10  $\mu\text{M}$ . In contrast, higher concentrations (25–100  $\mu\text{M}$ ) of genistein produced a dose-dependent decrease in cell growth when compared to untreated controls. These results are similar to those obtained by Martin et al. (1978) and Wang et al. (1997).

The cell proliferation studies (Figure 1) suggested that genistein acts via the estrogen receptor to enhance cell proliferation at low concentrations. Estradiol at concentrations of 0.2 nM and genistein at concentrations of 1  $\mu\text{M}$  to 10  $\mu\text{M}$  were observed to induce *pS2* gene expression (Figure 2). Additionally, we and others (Wang et al., 1996) have observed an increase in *pS2* mRNA expression by genistein at concentrations up to 50  $\mu\text{M}$  (data not shown). These data indicate that genistein can act as a weak estrogen agonist *in vitro* as measured by estrogen-dependent (*pS2*) gene expression (Hsieh et al., 1998).

## **GENISTEIN, ESTROGEN, AND BREAST CANCER—AN ISSUE OF DOSAGE AND TIMING**

Estrogen agonists also present a paradox with regard to breast cancer. It is generally accepted that estradiol will enhance growth of estrogen-dependent breast cancers (Lippman and Dickson 1989; Dickson, 1990). However, in certain animal models, estrogens are chemopreventive. For example, a combination of estrogen and progestin, given early, before the mammary gland differentiates, reduces the number of carcinogen-induced mammary tumors (Grubbs et al., 1985). Genistein can also act in a manner similar to estradiol in the rat mammary cancer model: when genistein (5 mg) is administered early in the rat's life and the mammary carcinogen dimethylbenz[a]anthracene (DMBA) is administered on day 56, genistein pretreatment reduced the number of carcinogen-induced tumors (Lamartiniere et al., 1995; Murrill et al., 1996). The authors suggest that genistein, like other estrogen agonists, enhances mammary gland growth and differentiation and ultimately reduces cell proliferation later in life. The differentiated mammary gland is protected against exposure to DMBA. Thus, timing of genistein treatment as well as timing of the carcinogen are critical as to whether these estrogenic chemicals act as chemoprevention agents or to stimulate growth of estrogen-dependent tumors. Similar studies have been conducted using dietary genistein (Fritz et al., 1998). One important question that remains to be answered is whether giving genistein after administration of the initiator will act to increase tumor number or estrogen-dependent tumor growth rate. Our research addresses the question as to whether genistein, when administered to mice implanted with estrogen-

# MCF-7 Tumor Growth Study



**Figure 2** The effect of estrogen pellet (2 mg) and dietary genistein (750 ppm) on MCF-7 tumor growth in athymic nude mice. MCF-7 human breast cancer cells were injected subcutaneously into four sites on the flanks of mice at  $1 \times 10^6$  cells per site. After tumors had formed, the mice were grouped to equalize tumor area and dietary treatment initiated. Experimental groups included negative control AIN93G (five mice, 15 tumors =  $n$ ), positive control implanted 2 mg estrogen pellet (five mice, 17 tumors =  $n$ ), and AIN93G + genistein 750 ppm (five mice, 17 tumors =  $n$ ). Data are expressed as change in tumor areas for each week of measurement. The treatment-week interaction is statistically significant ( $p < 0.0001$ ). Treatment means for each week are compared using the Least Significant Difference method.

dependent human breast cancer, will enhance growth of these existing human breast cancer cells. The following study addresses this issue.

We have designed studies to evaluate the effect of dietary genistein on the growth of estrogen-dependent human breast cancer (MCF-7) cells implanted into ovariectomized athymic mice. This is a model that has been used extensively to evaluate the tumorstatic action of tamoxifen (Gottardis et al., 1988). We selected a dosage of 750 ppm dietary genistein because this dietary dosage was able to induce estrogenic changes in both uterine and mammary tissue in ovariectomized rats; we hypothesized that genistein at this same level may enhance growth of implanted MCF-7 tumor cells in ovariectomized athymic mice. This hypothesis is supported by the *in vitro* data showing that low concentrations of genistein stimulated the growth of ER-positive human breast cancer cells. To evaluate the potential estrogenic effect of dietary genistein on tumor growth, we implanted MCF-7 cells at four sites in the flank region of ovariectomized athymic nude mice (Hsieh et al., 1998). Mice were fed the AIN93G diet. At the time of cell implantation, a pellet containing 2 mg of estradiol was inserted subcutaneously. Tumors appeared approximately 50 days later, at which point the mice were divided into three treatment groups with equal numbers of similar-size tumors. The estradiol pellet was removed from each animal. Control animals received AIN93G, positive control mice were reimplanted with pellets containing 2 mg estradiol and were fed AIN93G, and the third group of mice was fed the AIN93G diet containing 750 ppm genistein. MCF-7 cell tumors grew rapidly in the mice reimplanted with estradiol, and the mice were killed after three weeks of treatment because of the large size of the tumors. Tumors in mice fed AIN93G without estradiol implantation stopped growing. Mice fed 750 ppm of genistein had tumors that grew more slowly than those in the estradiol-treated mice, and tumor cross-sectional area reached that of the estradiol-treated mice after 12 weeks of genistein treatment. This indicates that genistein possessed sufficient estrogenic activity to stimulate growth of these estrogen-dependent tumors *in vivo* (Figure 2).

We believe that there are at least two dose-dependent mechanisms by which genistein alters cell growth: an estrogenic, growth-stimulatory mechanism that is active at low concentrations (100 nM to 1  $\mu$ M) and a growth-inhibitory mechanism that is active at concentrations above 20  $\mu$ M. We have conducted studies in rodents to determine plasma genistein levels when animals consume 750 and 3,000 ppm genistein in the AIN93G diet. We observed that total genistein (free, glucuronide conjugates, and sulfate conjugates) concentrations in plasma are approximately 1 and 6  $\mu$ M for the 750 ppm and 3000 ppm genistein diets, respectively (Santell et al., 1998). Most of the genistein in blood exists as the phase II glucuronide conjugate. It is generally accepted that the glucuronide (the major conjugate) of genistein is not biologically active.

In summary, genistein has several biological effects, including anti-proliferative effects on the growth of several cancer cell types, blockage of tyrosine phosphorylation, and stimulation of the growth of estrogen-dependent cells. Our recent work has focused on the estrogenic effects. There are numerous reports that genistein, like other estrogen agonists, can act as a chemopreventive agent to reduce the number of carcinogen-induced mammary tumors. However, genistein as an estrogen can also stimulate growth of estrogen-dependent tumors *in vivo*.

## CONCLUSIONS AND FUTURE RESEARCH

The issues presented in this chapter regarding genistein are complex. There is suggestive evidence that genistein may have numerous positive benefits on human health. These include reduction of bone loss in older women, reduction of risks for certain types of cancer, and other chemoprevention actions. However, we are far from being able to make clear recommendations regarding the consumption of high concentrations of highly enriched products containing the estrogenic soy isoflavones. Specifically, the efficacy of the soy isoflavones has not been clearly established in appropriate animal models and humans. Appropriate dosages cannot be established until efficacy has been verified. Once the dosage required for efficacy is determined, safety studies can then be designed to ensure that the effective dosages are safe for long-term consumption. It is also critical that sub-populations that may be more susceptible be identified and appropriate warnings for these populations at risk be made. One such population that may be at risk are women at high risk of acquiring or those already diagnosed with estrogen-dependent cancers. One cannot ignore the estrogenic activity of genistein and its potential to enhance growth of estrogen-dependent breast cancer in this sub-population.

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## **160 EFFECT OF GENISTEIN ON GROWTH OF HUMAN BREAST CANCER CELLS**

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## Cancer Prevention by Carotenoids and Curcumin

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### INTRODUCTION

**V**ARIOUS phytochemicals have been suggested to show preventive effects on oxidative damage-related diseases, including cancer. For example, carotenoids and curcumin are predicted to be effective in this aspect. In fact, various natural carotenoids were proven to have anticarcinogenic activity in animal studies. It is of interest that some of these carotenoids showed greater potency than  $\beta$ -carotene. Thus, these carotenoids ( $\alpha$ -carotene, lutein, zeaxanthin, lycopene,  $\beta$ -cryptoxanthin, phytoene, etc.), as well as  $\beta$ -carotene, may be useful for the purpose of cancer prevention. In the case of phytoene, the concept of bio-chemoprevention, which means a biotechnology-assisted method for chemoprevention, has been applied, and the establishment of mammalian cells producing phytoene was accomplished by the introduction of crtB gene, which encodes phytoene synthase. These cells were proven to acquire resistance against oxidative stress. We are planning to develop phytoene-containing animal foods in the future. It may be classified as a novel type of functional food that has preventive activity against oxidative damage-related diseases, as well as the ability to reduce the accumulation of oxidized substances, which are hazardous to human health. Curcumin has also been proven in animal experiments to inhibit carcinogenesis in various organs. The

combination of carotenoids and curcumin may increase the cancer chemopreventive activity.

## ANTI-CARCINOGENIC ACTIVITY OF NATURAL CAROTENOIDS

$\beta$ -carotene has been studied extensively as a promising anti-carcinogenic phytochemical. Recently, various natural carotenoids, besides  $\beta$ -carotene, were proven to have anti-carcinogenic activity in animal experiments. Some of them showed higher potency than  $\beta$ -carotene. For example,  $\alpha$ -carotene showed higher activity than  $\beta$ -carotene to suppress tumorigenesis in skin, lung, liver, and colon (Murakoshi et al., 1992; Narisawa et al., 1996).

In a skin tumorigenesis experiment, a two-stage mouse skin carcinogenesis model was used. Seven-week-old ICR mice had their backs shaved with electric clippers. From one week after initiation by 7,12-dimethylbenz[a]anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA) was applied twice a week for 20 weeks.  $\alpha$ - or  $\beta$ -carotene (200 nmol) was applied with each TPA application.  $\alpha$ -Carotene potency was greater than  $\beta$ -carotene. The percentage of tumor-bearing mice in the control group was 69%, whereas the percentages of tumor-bearing mice in the groups treated with  $\alpha$ - and  $\beta$ -carotene were 25% and 31%, respectively. The average number of tumors per mouse in the control group was 3.7, whereas the  $\alpha$ -carotene-treated group had 0.3 tumors per mouse ( $p < 0.01$ ). The  $\beta$ -carotene treatment resulted in 2.9 tumors per mouse, but the difference from the control group was not significant.

The greater potency of  $\alpha$ -carotene over  $\beta$ -carotene in the suppression of tumor promotion was confirmed by a second two-stage carcinogenesis experiment; i.e., 4-nitroquinoline 1-oxide (4NQO)-initiated and glycerol-promoted ddY mouse lung carcinogenesis model.  $\alpha$ - And  $\beta$ -carotene (at a concentration of 0.05%) or vehicle as a control was mixed as an emulsion into drinking water during the promotion stage. The average number of tumors per mouse in the control group was 4.1, whereas the  $\alpha$ -carotene-treated group had 1.3 tumors per mouse ( $p < 0.001$ ). The  $\beta$ -carotene treatment did not show any suppressive effect on the average number of tumors per mouse.

In a liver carcinogenesis experiment, a spontaneous liver carcinogenesis model was used. Male C3H/He mice, which have a high incidence of spontaneous liver tumor development, were treated for 40 weeks with  $\alpha$ - and  $\beta$ -carotene (at the concentration of 0.05%, mixed as an emulsion into drinking water) or vehicle as a control. The mean number of hepatomas was significantly decreased by  $\alpha$ -carotene treatment as compared with that in the control group; the control group developed 6.3 tumors per mouse, whereas the  $\alpha$ -carotene-treated group had 3.0 tumors per mouse ( $p < 0.001$ ). On the other hand, the  $\beta$ -carotene-treated group did not show a significant difference from the control group.

A short-term experiment to evaluate the suppressive effect of  $\alpha$ -carotene on colon carcinogenesis was carried out. The effect on *N*-methylnitrosourea (MNU) was examined in Sprague-Dawley (SD) rats; three intrarectal administrations of 4 mg in week one induced colonic aberrant crypt foci formation.  $\alpha$ - Or  $\beta$ -carotene (6 mg, suspended in 0.2 ml of corn oil, intragastric gavage daily) or vehicle as control were administered during weeks two and five. The mean number of colonic aberrant crypt foci in the control group was 62.7, whereas the  $\alpha$ - or  $\beta$ -carotene-treated group had 42.4 (significantly different from control group:  $p < 0.05$ ) and 56.1, respectively. Thus, the greater potency of  $\alpha$ -carotene compared with  $\beta$ -carotene was also observed in this experimental model.

Lycopene,  $\beta$ -cryptoxanthin, zeaxanthin, and lutein, as well as  $\alpha$ -carotene, were also proven to have higher anti-carcinogenic activity than  $\beta$ -carotene in various experimental systems. For example,  $\beta$ -cryptoxanthin showed significant anti-tumor promoting effect in a two-stage mouse skin carcinogenesis experiment at the dose of 40 nmol per painting, at which dose  $\beta$ -carotene did not show any suppressive effect (data not provided).

It is of interest that lycopene and  $\beta$ -cryptoxanthin have been found to activate the expression of the RB gene, a tumor suppressor gene, which might play an important role in anti-carcinogenic action of these carotenoids (Table 1).

In the case of phytoene, we applied a new concept: i.e., bio-chemoprevention. Valuable chemopreventive substances, including phytoene, may be produced in a wide variety of foods by means of biotechnology; this kind of new concept may be called bio-chemoprevention. As a prototype experiment, phytoene synthesis in animal cells was demonstrated (Nishino et al., 1992).

A phytoene synthase encoding gene, *crtB*, has already been cloned from *Erwinia uredovora*. We used this gene for the synthesis of the enzyme in animal cells. Mammalian expression plasmids, pCAcrtB, were constructed and transfected into mammalian cells either by electroporation or lipofection. NIH3T3 cells transfected with pCAcrtB showed the expression of a 1.5 kb mRNA from the *crtB* gene as a major transcript. Those transcripts were not present in the cells transfected with the vector alone.

For analysis of phytoene by HPLC, the lipid fraction, including phytoene, was extracted from cells ( $10^7$ – $10^8$ ). The sample was subjected to HPLC (col-

TABLE 1. Effect of lycopene and  $\beta$ -cryptoxanthin on RB gene expression.

Treatment	Relative Expression Rate (%)
+ Lycopene	405
+ $\beta$ -Cryptoxanthin	284

Carotenoids were added into cell culture medium at the concentration of 10  $\mu$ M for 24 hours.

umn: 3.9 by 300 mm, Nova-Pak HR, 6m C18, Waters) at a flow rate of 1 ml/min. To detect phytoene, UV absorbance of the eluate at 286 nm was measured by a UV detector (JASCO875).

Phytoene was detected as a major peak in an HPLC profile of NIH3T3 cells transfected with pCcrtB, but not in control cells. Phytoene was identified by UV- and field desorption mass-spectra.

Because lipid peroxidation is thought to play a critical role in tumorigenesis, it was suggested that the antioxidant activity of phytoene may play an important role in its mechanism of anticarcinogenic action. The level of phospholipid peroxidation induced by oxidative stress in cells transfected with pCAcrtB or with vector alone was compared. The phospholipid hydroperoxidation level in the cells transfected with pCAcrtB was significantly lower than that in the cells transfected with vector alone. Thus, anti-oxidative activity of phytoene in animal cells was confirmed.

Thus, phytoene may become a valuable factor in animal foods to reduce the formation of oxidized oils, which are hazardous to health, as well as to maintain freshness, resulting in the maintenance of high quality of foods. Furthermore, phytoene-containing foods may be valuable for cancer prevention, because phytoene is recognized as an anticarcinogenic substance. Thus, it may become one of the fundamental methods for bio-chemoprevention and especially for the development of novel functional animal foods.

## ANTI-CARCINOGENIC ACTIVITY OF CURCUMIN

Curcumin is the major yellow pigment in turmeric, which is widely used as a spice and coloring agent in foods, such as curry. The anti-carcinogenic activity of curcumin was also extensively studied. For example, we examined the effect of curcumin on the tumor-promoting process of two-stage carcinogenesis of mouse skin (Satomi et al., 1998). The percentage of tumor-bearing mice in the control group was 96%, whereas the percentage of tumor-bearing mice in the groups treated with curcumin was 7%. The average number of tumors per mouse in the control group was 11.2, whereas the curcumin-treated group had 0.1 tumors per mouse ( $p < 0.001$ ). Recently, we also found that nitric oxide (NO) generator-induced tumorigenesis in mouse skin was suppressed by oral administration of curcumin (Table 2).

The mechanism of action of curcumin was investigated, and it was found that it showed scavenging activity for various reactive oxygen species, including NO. In addition to scavenging activity for free radicals, curcumin was found to interact with  $\text{Ca}^{2+}$ -calmodulin complex. Thus, multiple mechanisms should be considered with respect to the anti-carcinogenic action of curcumin.

TABLE 2. Effect of curcumin on skin carcinogenesis in SENCAR mice treated with NO and TPA.

Group	(n)	Tumor-Bearing Mice (%)	Average Number per Mouse
Control	(15)	100 <sup>a</sup>	7.33 <sup>b</sup>
+ Curcumin	(15)	53.3 <sup>a</sup>	3.00 <sup>b</sup>

<sup>a</sup>p < 0.05.

<sup>b</sup>p < 0.05.

Mice were treated with NOR1 (tumor initiator, 390 nmol, once), an NO donor, and TPA (tumor promoter, 1.7 nmol, twice a week for 20 weeks). Curcumin (0.0025% in drinking water) was administered during whole period of the experiment; i.e., from one week before the tumor initiation to the end of the tumor-promoting period.

## CONCLUSION

Carotenoids and curcumin are common natural antioxidants, which seem to be useful for the development of functional foods for cancer prevention. Combination of carotenoids and curcumin may improve the cancer prevention capabilities. We should examine such possibilities and investigate the mechanisms of action more precisely.

## ACKNOWLEDGEMENTS

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# Alfalfa Saponins: Chemistry and Application

WIESLAW A. OLESZEK

## INTRODUCTION

**T**HREE are two major reasons why alfalfa saponins have been extensively studied by several research groups recently. These include nutritional aspects of saponins which occur in one of the most popular legume pastures and the pharmacological properties of these triterpene glycosides.

There is evidence that performance of monogastric animals is correlated to the saponin concentration in the diet containing alfalfa (Cheeke, 1983; Cheeke, et. al., 1977; Pedersen et al., 1972; Price et al., 1987; Reshef et al., 1976). Growth reduction was observed when the concentration of saponins in the diet increased. The mechanism of this harmful effect is not fully understood, but its most important impact is on the taste of the pasture. Alfalfa saponins are bitter, astringent, and throat-irritating compounds, which was proved in taste trials with laboratory staff volunteers, using pure saponins isolated from alfalfa aerial parts (Oleszek et al., 1992). If similar effects are found in animals, the palatability of an alfalfa-based diet may be lowered and may adversely affect feed intake. This correlates with the previous finding of Cheeke (1983), who recorded effects on feed intake to be one of the main, if not the major, mechanisms by which legume saponins exerted their growth-depressing effects. Thus, it is rather the taste of saponins and not their toxicity that is responsible for their growth-retarding activity. Addition of 1% and 1.2% alfalfa top saponins in the diet or 40% alfalfa seeds reduced both plasma and aortic tissue cholesterol levels, without any evidence of toxic symptoms (Malinow et al., 1981a).

Once swallowed, saponins may react with the membranes of the digestive

tract, especially with the small intestine walls. This is due to their abilities to bind membrane sterols. The hydrophobic aglycone of the saponin molecule penetrates the lipid bilayer and may specifically interact with other membrane components, such as cholesterol, producing conducting channels and making the membrane leaky. This increased cell permeability may primarily influence the absorption of nutrients, but perhaps also may influence the absorption of allergens, xenobiotics, and other toxic dietary components. In this respect, alfalfa saponins are the most potent depolarizer among several other types of saponins tested (Gee et al., 1989).

Different effects can be observed when ruminants are fed saponin-containing diets. Intraruminal administration of alfalfa saponins up to the concentration of 4% in feed dry matter resulted in reduction of rumen nutrient degradation and microbial fermentation. In the presence of saponins, fractional digestive coefficients of organic matter, hemicellulose, cellulose, and nitrogen were reduced in stomach environment, but increased in the small intestine, which in fact improved efficiency of nutrient utilization (Lu and Jorgensen, 1987). But some authorities claim that the rumen microflora is able to utilize only the carbohydrate parts of saponins (Gutierrez and Davis, 1962) and resulting prosapogenins or aglycones show increased toxicity, which is especially harmful for the microflora of rumen and to the digestive functions of digestive system (Klita et al., 1996).

The role that alfalfa saponins may play in ruminant bloating, due to their foaming properties, has not been fully understood. While some data indicate that saponins bear responsibility for bloating (Lindahl et al., 1957; Marten et al., 1990), others clearly demonstrate that there is no correlation between saponin concentration and bloating incidence (Majak et al., 1980, 1995). These are rather soluble proteins (Howarth et al., 1973) and chlorophyl or some other components are responsible for this effect (Majak et al., 1995).

Alfalfa saponins are also of interest due to their ability to lower serum cholesterol levels. It is generally accepted that elevated plasma cholesterol is a significant risk factor in the etiology of cardiovascular disease. Extensive work performed by Malinow and co-workers (Malinow et al., 1977, 1978, 1981b, 1987, 1992) indicated that alfalfa saponins may provide a useful means of dietary management of plasma cholesterol in man. In experiments with higher primates fed 1% isolated alfalfa root or 0.6% alfalfa top saponins, no toxicity was observed, while regression of aortic and coronary atherosclerosis was evident. It is generally believed that saponins bind dietary cholesterol and limit its absorption, but they can also alter cholesterol metabolism by interfering with enterohepatic bile acid and salt circulation, leading to an increased fecal output, and the feed-back effect is an increase in cholesterol conversion into bile acids. This principle is presently clinically exploited in the treatment of hypercholesterolemic patients. The best results have been obtained if hypcholesterolemic saponins are administered with good quality

dietary fiber. This has been demonstrated successfully using saponins from *Saponaria officinalis*, *gypsophila* saponins, and *quillaja* saponins.

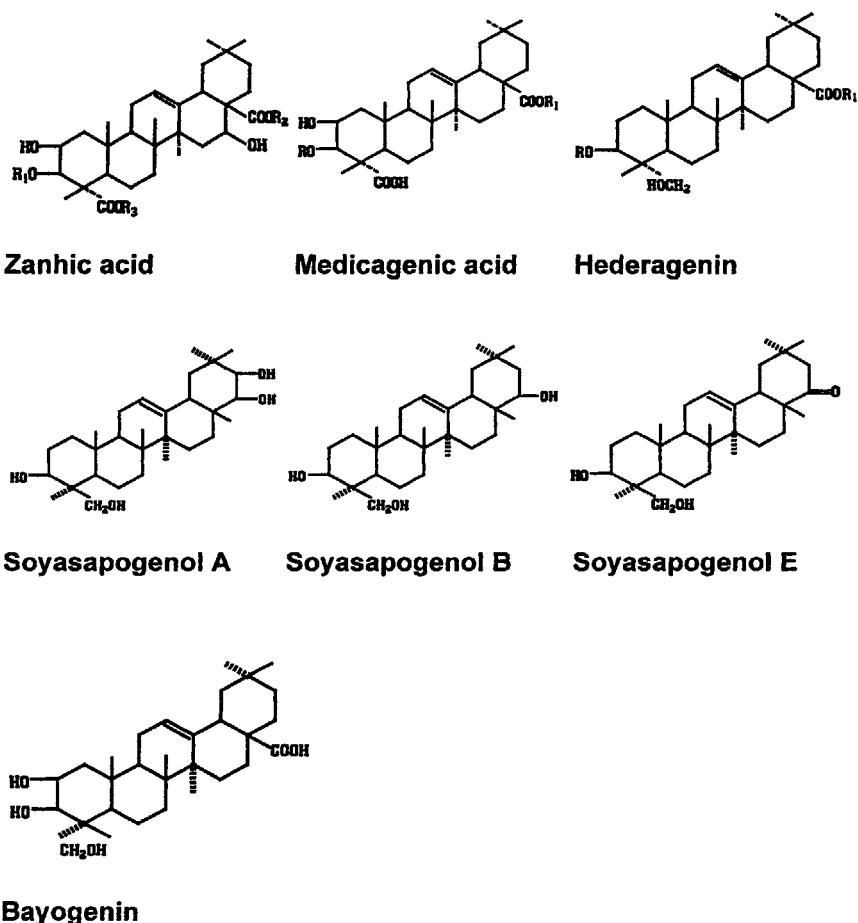
The weak point of early, and some recent, alfalfa saponin application studies was the fact that crude saponin fractions were very poorly defined in their composition and some important saponin components were totally ignored. This was due to the fact that the only means of the control of saponin quality were biological tests (*Trichoderma viride* growth and hemolytic potential), which were providing very limited information regarding saponin composition, and, in the case of alfalfa saponins, they could recognize only medicagenic acid and hederagenin glycosides (often called the "biologically active" fraction). They did not consider structure-dependent activities, did not register the presence of such important components as zanhic acid and soyasapogenol glycosides, and did not recognize seasonal variations of saponins in plant material (Oleszek, 1996). Thus, the aim of this chapter is to summarize the actual knowledge of the chemistry, biological activity, and environmental influence on alfalfa saponins and their potential use.

## AGLYCONES OF ALFALFA SAPONIN

Agllycones are a non-sugar part of saponin molecules. Generally, they do not occur in alfalfa in a free form, but as differently glycosylated compounds. Multiplicity of glycosylation patterns results in a mixtures of saponins composed with a great number of individual glycosides. The sugar molecules are attached to the aglycone mostly at the 3-OH position, giving rise to monodesmosides (Greek desmos = chain). Bidesmosides have also been shown to commonly occur, and this utilizes the 3-OH and 22-OH positions for glycosylation of soyasapogenols or the 3-OH and 28-OH positions for medicagenic acid, zanhic acid, hederagenin, and bayogenin. Tridesmosides, glycosylated at 3-OH, 23-OH, and 28-OH, have also been reported (Oleszek et al., 1992).

Different parts of the alfalfa plant show characteristic saponin patterns, both in aglycone and glycosidic structures. Aglycones of alfalfa are all composed exclusively of a triterpene skeleton with different functional groups substituted. These include medicagenic acid, zanhic acid, hederagenin, soyasapogenols, and bayogenin (Figure 1). From the group of soyasapogenols, only soyasapogenols A, B, and E seem to be a natural forms. In acid hydrolysates of alfalfa saponins, additionally, soyasapogenols C, D, F, and G can be found, but these are being regarded as artifacts arising from soyasapogenol B (Jurzysta, 1984).

As early as 1959, Livingston reported the presence of a new compound called lucernic acid (Livingston, 1959). But as recently proved, this compound was also an artifact, the 13→28 lactone formed by acid-catalyzed cyclization



**Figure 1** Chemical structures of the aglycones of alfalfa saponins.

of the  $\gamma,\delta$ -unsaturated acid, which in this case proved to be zanhic acid ( $16\alpha$ -hydroxymedicadic acid) (Massiot et al., 1988b).

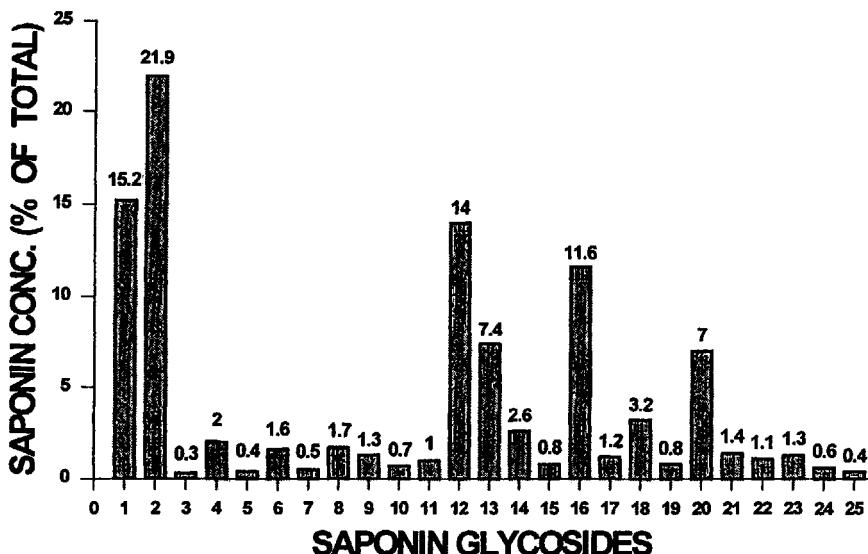
## ROOT SAPONINS

Roots of alfalfa are the plant organs richest in saponins, and, thus, most of the work has been concentrated on this fraction. Nine dominant glycosides were identified by Oleszek and co-workers (1990), a number were reported by Timbekova and colleagues (Timbekova 1996), and several glycosides were reported by Massiot (1988a). All of these data were reviewed in detail (Oleszek,

TABLE 1. The chemical structures of saponins identified in alfalfa roots.

Compounds	R	R <sub>1</sub>
<i>Medicagenic Acid Glycosides</i>		
1	Glu	H
2	Glu	Glu
4	Glu(1→3)	Glu
5	Glu	Rha(1→2)-Ara
7	Rha(1→2)-Glu(1→2)-Glu	H
10	Glu(1→2)-Glu(1→2)-Glu	Glu
11	Rha(1→2)-Glu(1→2)-Glu	Glu
12	Glu	Xyl(1→4)-Rha(1→2)-Ara
15	GluA (estrified CH <sub>3</sub> )	Xyl(1→4)-Rha(1→2)-Ara
18	Glu(1→2)-Glu	Xyl(1→4)-Rha(1→2)-Ara
20	Glu A	Xyl(1→4)-Rha(1→2)-Ara
22	Glu(1→2)-Glu(1→2)-Glu	Xyl(1→4)-Rha(1→2)-Ara
25	Glu(1→2)-Glu(1→2)-Glu	Xyl(1→4)-Rha(1→2)-Ara Api(1→3)
<i>Hederagenin Glycosides</i>		
3	Glu(1→2)-Ara	H
6	Glu(1→2)-Ara	Glu
8	Ara(1→2)-Glu(1→2)-Ara	H
13	Ara(1→2)-Glu(1→2)-Ara	Glu
<i>Soyasapogenol Glycosides</i>		
<i>Soyasapogenol A</i>		
14	Rha(1→2)-Gal(1→2)-GluA	Rha
<i>Soyasapogenol B</i>		
9	Rha(1→2)-Gal(1→2)-GluA-(estrified CH <sub>3</sub> )	
16	Rha(1→2)-Gal(1→2)-GluA	
<i>Soyasapogenol E</i>		
19	Rha(1→2)-Gal(1→2)-GluA	
<i>Bayogenin</i>		
17	Glu(1→2)-GluA	Gal
<i>Zanhic Acid Glycosides</i>		
21	Glu(1→2)-Glu(1→2)-Glu	Xyl(1→4)-Rha(1→2)-Ara
23	Glu(1→2)-Glu(1→2)-Glu	Xyl(1→4)-Rha(1→2)-Ara Api(1→3)
24	Not established (Glu + Ara + Rha + Xyl)	

1996). Very recently, extensive work has been performed by Bialy (1998), who isolated and identified 25 root glycosides, including 13 glycosides of medicagenic acid, three glycosides of zanhic acid, four compounds with hederagenin as aglycone, one glycoside of soyasapogenol A, two saponins of soyasapogenol B, one of soyasapogenol E, and one glycoside of bayogenin. Their structures are presented in Table 1.



**Figure 2** Concentration of individual alfalfa root saponins expressed as the percentage of total, evaluated from the isolation efficiency. Saponin numbers 1–25 are in agreement with the chemical structures presented in Table 1.

Analyzing these nice sequences of glycosylation of medicagenic acid, some regularities can be immediately noticed. The simplest structure, 3-O-Glu, can be glucosylated at C-28 either by another glucose or by the sequence-Ara-Rha-Xyl and in one case with extra sugar apiose. The chain at C-3 can also be made longer in all cases with another glucose, and then with a terminal glucose or arabinose. Only two compounds are substituted at C-3 with glucuronic acid. The same sequences of sugars can be found at zahnic acid glycosides (compounds 21 and 23). The absolute lack of zanthic acid glycosides having shorter sugar chains, as well as their low concentration in the roots, may indicate that compounds 21 and 23 are oxidation products at C-16 of appropriate medicagenic acid glycosides (compounds 22 and 25, respectively), which may prove their phylogenetic relationship.

Glycosylation of hederagenin at C-3 starts with arabinose, and this chain can be made longer by the attachment of glucose and another arabinose. The C-28 can be glucosylated exceptionally with glucose. The soyasapogenol glycosides possess the same sugar sequence at C-3 and differ only by the substitutions at ring five in aglycone molecule.

Based on extraction efficiency, only six compounds can be recognized as definitely dominant in the mixture (Figure 2). These include four glycosides of medicagenic acid (1:15.2% of total saponin, 2:21.9%, 12:14%, and 20:7%), one glycoside of hederagenin (13:7.4%), and one of soyasaponin I (16:11.6%).

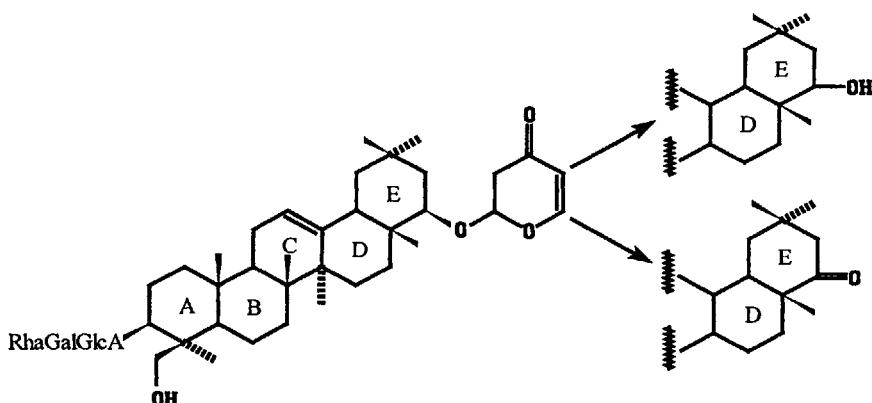
Great numbers of the compounds are present in trace amounts (1% of the total and lower), and some in the concentration between 1 and 3% of total. These findings generally correlate with previous data obtained with liquid chromatography (HPLC) for Boja variety (Nowacka and Oleszek, 1994), where dominant medicagenic acid compounds were the same (1:5.7% of total, 2:15.6%, 12:16.8%, 20:33.9%), but the mutual proportions of these glycosides were totally different. Soyasaponin I occurred at the trace level of 2.4%. These findings clearly show that composition of root saponins can qualitatively be the same in different plant material (different varieties, stage of growth, environmental influence), but quantity of individual compounds in the mixture can show substantial variation. The most important variation is that of compound **1**. This compound inhibits *Trichoderma viride* in 50% ( $IA_{50}$ ) at the concentration of 0.16 mg/100 ml, while to obtain the same inhibition, 3.3 mg of **2**, 1.35 mg of **12**, and 4.75 mg of **20** is required (Oleszek et al., 1990). Glycoside of hederagenin has  $IA_{50}$  at 8.2 mg/100 ml and soyasaponin I show no activity. Thus, it is clear that a concentration of 3-O-Glu of medicagenic acid is crucial for determination of biological activity of root saponin mixture. The same is true in the case of hemolytic activity (Oleszek, 1990), activity against medically important yeasts *Candida*, *Cryptococcus*, *Torulopsis* (Polacheck et al., 1986) and plant pathogens *Scelotium rolfsii*, *Fusarium oxysporum* ssp. *lycopersici*, *Rissoctonia solani*, *Trichoderma viride*, *Aspergillus niger*, *Pythium aphanidermatum* (Levy et al., 1989), *Cephalosporium gramineum*, and *Gaeumannomyces graminis* (Martyniuk et al., 1995).

This variation is also crucial in determination of saponins in plant material. As previously shown, concentration of saponins in alfalfa roots can reach the level of 5% in dry matter, or even higher, when determined with *T. viride* or hemolytic tests; when measured with IIPLC, this concentration is two times lower (Nowacka and Oleszek, 1994). The same is true when saponins are determined in germinating alfalfa seeds and seedlings (see below).

The above consideration clearly shows that any saponin mixture used in biological tests needs to be thoroughly characterized if we expect the data obtained in different experiments to be repeatable.

## SEED SAPONINS

Saponins from alfalfa seeds were not extensively studied with respect to their glycosidic structures. In the early work performed by Jurzysta (1973), it was documented that seed saponins consist of four glycosides (based on thin layer chromatography, TLC) with one of them being dominant. Analysis of hydrolysis products showed the presence of soyasapogenols B, C, and E and four dominant sugars, including glucose, galactose, rhamnose, and glucuronic acid. Thus, it was evident that alfalfa seeds contain soyasapogenol



**Figure 3** Decomposition of chromosaponin I into the glycoside of soyasapogenol B (-OH) or into the glycoside of soyasapogenol E (=O).

B glycosides like the seeds of most of the leguminous species (Price et al., 1987). Liquid chromatographic analysis showed that seeds of Boja cultivar contain only one saponin detectable with this technique. From the retention time, this was identified as soyasaponin I, occurring at the concentration of 2.12  $\mu\text{mol/g}$  of dry matter. This concentration was quite stable during the germination and early seedling growth (Oleszek, 1998). A similar concentration of soyasaponin I can be found in aerial parts of mature alfalfa plants (Nowacka and Oleszek, 1994). However, this is not soyasaponin I but rather chromosaponin I, which, according to recent reports, is a genuine compound occurring in the germinating seeds of some legumes (Massiot et al., 1992; Kudou et al., 1993; Tsurumi et al., 1992). Soyasaponin I is thought to be an artifact from its DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) conjugate (Figure 3), which may degrade easily during the sample preparation (Massiot et al., 1992). Two degradation paths are possible that generate either soyasapogenol B or soyasapogenol E prosapogenins. This may explain the lack of clarity regarding the status of soyasapogenol E glycosides: Are these natural forms or rather artifacts? Soyasapogenol E can be found in the mixture of sapogenols together with soyasapogenols C, D, and F when soyasapogenol B glycosides are hydrolyzed in the presence of water. The DDMP-conjugated saponins are readily soluble in water, while soyasaponin I in a free form precipitates from alcohol-water solutions in a crystalline form. They have been reported in many kinds of legumes, such as chickpea (*Cicer arietinum* L.), scarlet runner bean (*Phaseolus coccineus* L.), kidney bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), mung bean [*Vigna mungo* (L.) Hepper], and cowpea [*Vigna sinensis* (L.) Hassk].

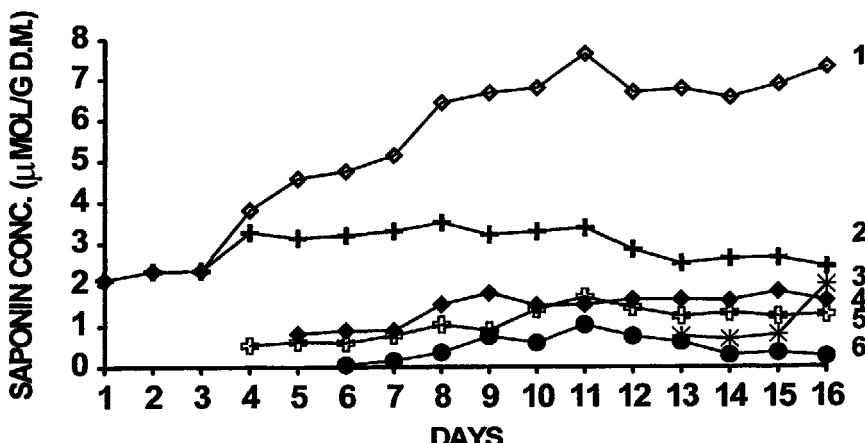
As shown in many studies, soyasaponin I occurring in soybean and other legume seeds shows no cytotoxicity or mutagenicity, has no haemolytic and

antifungal activity (Oleszek, 1996), is inactive in relation to the intestine membranes, and in *in vitro* tests does not change the rat small intestine transmural potential difference (Gee et al., 1989). However, soyasaponin I is able to bind cholesterol, which is believed to be a basic feature for saponins to show most of the mentioned activities. Binding cholesterol without any side effects makes soyasapogenol saponins good candidates for removing cholesterol from the diet and bile salts from the digestive system for hypercholesterolemia treatment. Saponin-cholesterol complexes are poorly soluble in water (Jurzysta, 1973) and are excreted with feces.

## SAPONINS FROM ALFALFA SEEDLINGS

The human consumption of alfalfa products is generally low, but in some countries alfalfa sprouts are being used as a green salad (Oakenfull, 1980). Early studies of alfalfa seedlings showed that they can be extremely rich in saponins. Rapid synthesis of biologically active saponins in germinating seeds and sprouts was reported (Pedersen, 1975; Gorski et al., 1991). As measured by bioassays, the concentration of saponins in seedlings reached a very high level—up to 8 to 10% of dry matter (Fenwick and Oakenfull, 1983; Price et al., 1987; Gorski et al., 1991). Reinvestigation of the germination process performed with analytical HPLC procedure provided a completely different picture of saponin synthesis in alfalfa seedlings (Oleszek, 1998). For the first three days of the germination process, only soyasaponin I was detected at the level of 2.12  $\mu\text{mol/g}$ , the same as in genuine seeds. On the fourth day of germination, the synthesis of medicagenic acid glycosides started (Figure 4). The first compound of this group was monodesmosidic 3-O-glucoside of the medicagenic acid (**1**). Its concentration was increasing gradually and was established at the level of 1.2 to 1.3  $\mu\text{mol/g}$  after the eleventh day of germination. On the fifth day, bidesmosidic 3GlcA,28AraRhaXyl medicagenic acid (**20**) was observed, and on the sixth day, 3Glc,28Glc medicagenate (**2**) was observed. Their concentrations ranged from 0.82 to 1.8 and from 0.06 to 1.04  $\mu\text{mol/g}$ , respectively, and after 10 days showed quite stable levels. Zanhic acid tridesmoside (3GlcGlcGlc,23Ara,28AraRhaXylApi 16-OH medicagenate), one of the dominant compounds of alfalfa tops (see below), appeared for the first time after 12 days of seedling growth at the concentration that was comparable to medicagenic acid glycosides.

Total saponin content in the seedlings showed gradual increase during first eight days, due to the new compounds being synthesized, to the level of 6  $\mu\text{mol/g}$ , and, afterward, this remained quite stable. This level corresponds to the total saponin concentration of 0.6% in dry matter and differs substantially from the previous reports indicating the concentration of saponin in alfalfa seedlings at 8 to 10% of dry weight. The assayed concentration in seedlings



**Figure 4** Concentration of saponins during alfalfa seed germination and early seedling growth: 1, total saponin concentration; 2, soyasaponin I; 3, zanhic acid tridesmoside; 4, 3Glu,28Glu medicagenic acid; 5, 3Glu medicagenic acid; 6, 3GluA,28AraRhaXyl medicagenic acid.

is not much different from the level in mature alfalfa plants of varieties recognized as intermediate in saponin level.

These findings clearly show again that the type of analysis is very important in the determination of real concentration and quality of plant saponin preparations. Biological tests used for determination of saponins in alfalfa seedlings produce results that were drastically overestimated. Similar overestimations occurred when biological tests were used for alfalfa root saponin determination (Nowacka and Oleszek, 1994). The reason for these estimates was the same in both cases—variation in the concentration of the highly active 3-O-glucoside of medicagenic acid, which was present both in root and seedling material.

## ALFALFA AERIAL PARTS

Saponins from aerial parts of alfalfa have predominantly bidesmosidic structure. Dominant saponins of medicagenic acid are the same that occurred in substantial concentration in the roots (Massiot et al., 1991; Oleszek et al., 1992; Nowacka and Oleszek, 1994). This includes compounds **12**, **18**, and **20**, of which saponin **20** (3GluA,28AraRhaXyl medicagenic acid) is definitely dominant (Table 2). It is usually occurring together with its dextro derivative (3GluA,28AraRha, **27**). Cholesterol precipitable alfalfa top saponins may contain as much as 60 to 70% of compound **20** (Oleszek, 1991). Thus, when top saponins are being determined with biological tests, the results show only medicagenic acid fraction, predominated with saponin **20**. Remaining saponins

TABLE 2. The chemical structures of saponins identified in alfalfa aerial parts.

Compound	R	R <sub>1</sub>	R <sub>2</sub>
<i>Medicagenic Acid Glycosides</i>			
12	Glu	Xyl(1-4)Rha(1-2)Ara	
18	Glu(1-2)Glu	Xyl(1-4)Rha(1-2)Ara	
20	GluA	Xyl(1-4)Rha(1-2)Ara	
26	H	Xyl(1-4)Rha(1-2)Ara	
27	GluA	Rha(1-2)Ara	
<i>Soyasapogenol B Glycosides</i>			
16	Rha(1-2)Gal(1-2)GluA	-OH, -H	
28	Rha(1-2)Glu(1-2)GluA	-OH, -H	
29	Glu(1-2)GluA	-OH, -H	
<i>Soyasapogenol E Glycoside</i>			
19	Rha(1-2)Gal(1-2)GluA	=O	
<i>Zanhic Acid Glycosides</i>			
30	Glu(1-2)Glu(1-2)Glu	Api(1-3)Xyl(1-4)Rha(1-2)Ara	Ara
31	Glu(1-2)Glu(1-2)Glu	Xyl(1-4)Rha(1-2)Ara	Ara

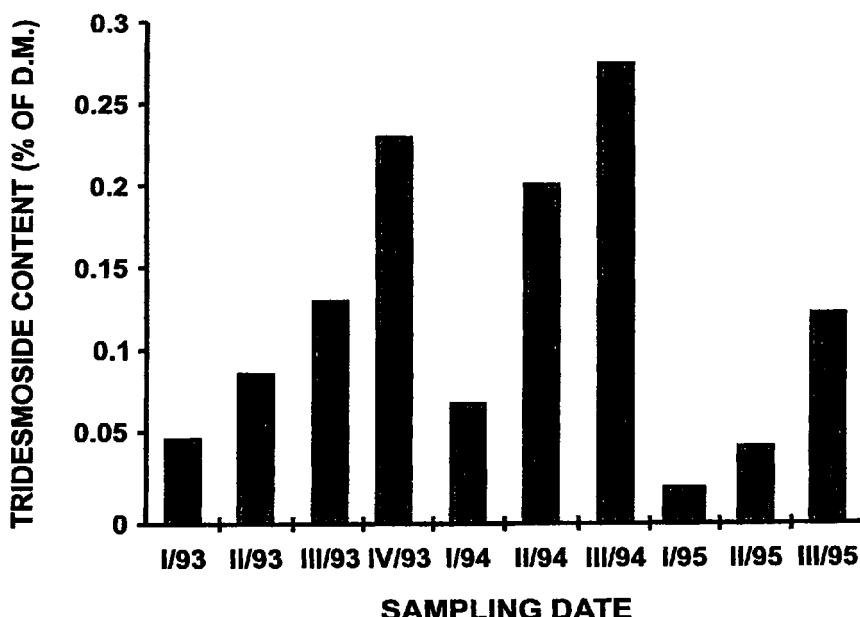
nins, such as soyasapogenol and zanhic acid glycosides, are not recognized by these tests. This is why, in the case of aerial parts, HPLC determination gives higher saponin concentration than the *T. viride* test (Nowacka and Oleszek, 1994).

As shown above, soyasaponin does not influence the *T. viride* growth and does not show hemolytic activity. It can be found in alfalfa aerial parts and determined with HPLC procedure. Its concentration may be as high as 0.2 to 0.3% of dry matter and can make up 20% of total saponins present.

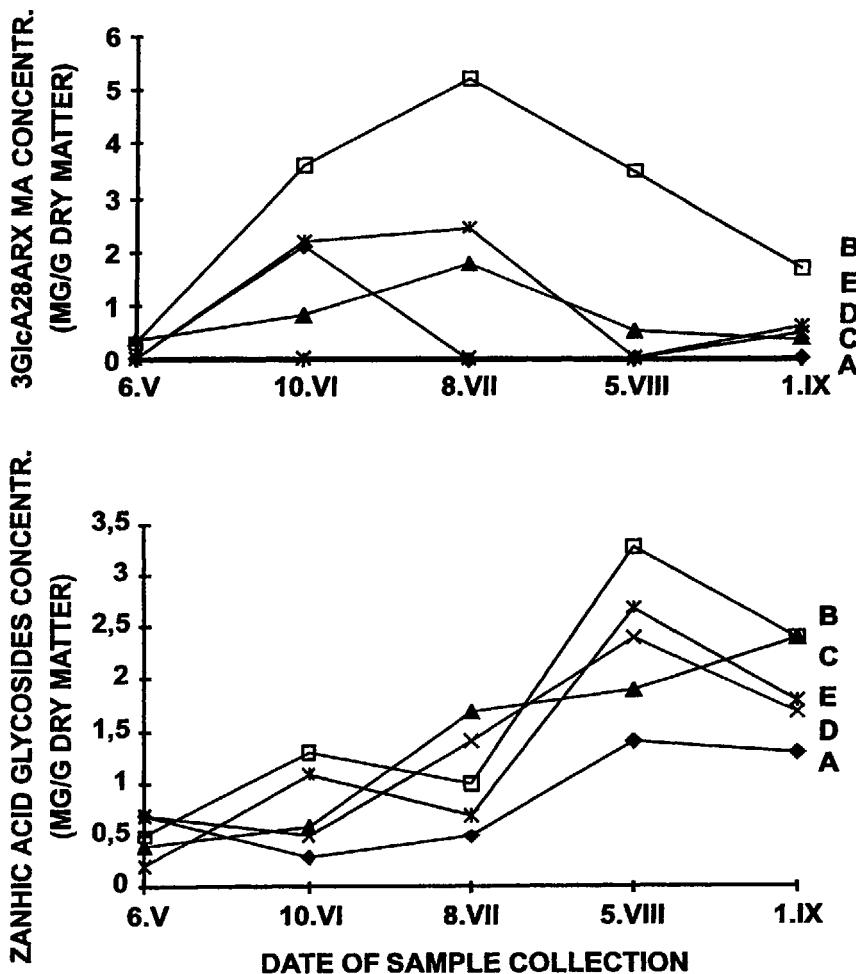
Aerial parts also contain substantial amounts of zanhic acid glycosides. For a long time, these compounds were not known in alfalfa, and in the preparation of saponin mixture they were being lost. This was due to the technique of purification used. In early studies of saponins, the first step of their purification from alcohol-water extract was evaporation of alcohol and liquid-liquid extraction with butanol. In this step, highly polar compounds, including carbohydrates, remained in the water, and saponins were extracted to butanol, which on evaporation resulted in a brownish, syrup-like, crude saponin fraction. It was further purified by different means, including cholesterol or lead acetate precipitation. Unfortunately, in the liquid-liquid extraction, some highly polar saponin components did not go readily to butanol but rather stayed in water together with carbohydrates. Separation of highly polar saponins from carbohydrates became possible after application of solid-phase extraction on C18 reversed phase supports (Oleszek, 1988). This technique allowed us to separate two novel compounds, which by means of spectral analyses (FAB-MS, NMR) were described as dominant zanhic acid tridesmoside and, occurring in trace

amounts, its deapio-derivative (Oleszek et al., 1992). Tridesmoside does not inhibit *Trichoderma viride*, shows just a trace of hemolytic activity and cannot be detected with these biological tests. To be able to trace this compound in plant material, an HPLC procedure for its determination has been developed (Nowacka and Oleszek, 1992).

Three-year field experiments (1993–1995) performed at an experimental farm at the Institute of Soil Science and Plant Cultivation, Pulawy, Poland, with nine alfalfa varieties showed that the concentration of zanhic acid tridesmoside changed during the growing season (Nowacka, 1998). The data in Figure 5 represent average values for nine following varieties: Boja (Polish var.), Europe (French var.), Canadian (Canadian population, seeds available on Polish market), Lodi (Italian var.), Magali (French var.), Natsuwakaba (Japanese var.), Radius (Polish var.), S69+ (French var.), Tula (Polish var.). They clearly show that the lowest concentration (0.02–0.07% d.m.) can usually be found in the first cut of alfalfa in spring (May/June). It increases gradually during the growing season to be the highest (0.15–0.3% d.m.) in the last cut (middle of September). Similar results were obtained in the experiment performed in Lodi, Italy, with five varieties: Boreal, La Rocca, Lodi, Romagnolo, and Prosementi (Figure 6). Again, the highest concentration of zanhic acid glycoside was found at the end of the growing season. Italian experiments showed

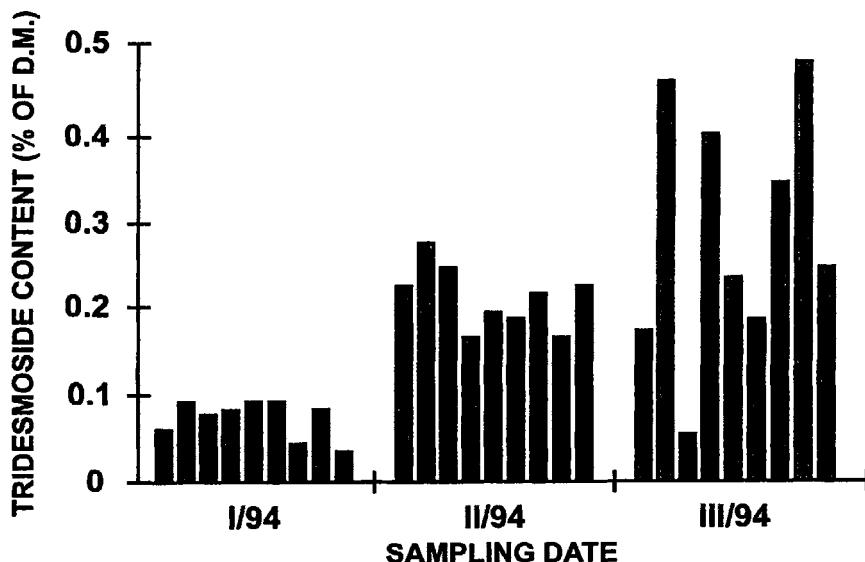


**Figure 5** Zanhic acid tridesmoside concentration—average values for nine alfalfa varieties in different cuts (I–IV) of the three growing seasons (93–95).



**Figure 6** Comparison of the changes in the concentration of 3GluA,28AraRhaXyl medicagenic acid (upper) and zanhic acid tridesmoside (lower) in five alfalfa varieties (A–E) during the growing season: A—Boreal; B—Romagnolo; C—La Rocca; D—Lodi; E—Prosementi.

additional lack of correlation between the concentration of zanhic acid tridesmoside and 3GluA,28AraRhaXyl medicagenic acid, the dominant medicagenic acid glycoside. Maximum zanhic acid concentration started at the moment when the amount of medicagenic acid glycoside drastically decreased (Tava et al., in press). Such a tendency occurred, but not so clearly, in nine Polish varieties, where concentration of zanhic acid during one season correlated with total saponin amount measured with *T. viride* test and during two other seasons did not. But differences in zanhic acid concentration were evident



**Figure 7** Changes in the concentration of zanhic acid tridesmoside in nine alfalfa varieties during the growing season of 1994. Varieties (from left to right): Boja, Europe, Canadian, Lodi, Magali, Natsuwakaba, Radius, S69+, Tula.

between different growing seasons. In the first and second year of alfalfa stand utilization, concentration was high, while in a third year, it was substantially lower (Figure 5). It is not clear if these differences are the result of the alfalfa age or rather they represent the influence of environment on zanhic acid synthesis (total saponin concentration measured with *T. viride* did not drop so drastically). Differences in zanhic acid tridesmoside were also evident between varieties. As shown in Figure 7, the concentrations in the first and second cut were quite similar for nine varieties. In a third cut in Canadian variety, the concentration of zanhic acid was 0.07% in dry matter, while in the three other varieties (Europe, Lodi, and S69+), the amount of this compound was between 0.4 and 0.5% in dry matter. It is worth emphasis because French varieties (Europe and S69+) are generally known as high saponin cultivars, and high zanhic acid concentration seems to be correlated with this elevated total saponin content.

The above data clearly show that zanhic acid tridesmoside is a very important ingredient of alfalfa top saponins that is not detected with standard biological tests, which concentration is changing with variety, with the season, and with the time of sampling. It is very important for nutritionists and pharmacologists to consider all these facts in their efforts on saponin utilization.

## BIOLOGICAL ACTIVITY OF ALFALFA SAPONINS

Dominant, individual alfalfa saponins (Table 1) have been tested in a number of biological tests. These include hemolysis, antifungal, mutagenic, cytotoxic, membrane depolarizing, allelopathic, insecticidal, and herbicidal activities. The allelopathic and pesticidal activities have been recently reviewed in some detail (Oleszek, 1996; Oleszek et al., 1999). Others that are more closely related to nutritional/pharmaceutical properties will be discussed in this paragraph.

### HEMOLYSIS

The characteristic feature of many saponins is their ability to lyse erythrocytes. From the nutritional point of view, this feature is not very important as it is unlikely that saponins cross the intestinal membranes and enter the bloodstream. But, hemolysis has been successfully used as a biological test for saponin determination in plant material. From among the alfalfa saponins, only medicagenic acid itself and its 3-O-glucoside show high hemolytic activity (HI between 10,000 and 20,000; hemolytic index understood as the volume in milliliters of 2% v/v solution of cows blood in isotonic buffered solution that could be fully hemolyzed by 1 g of saponins). Bidesmosidic medicagenic acid glycosides show much lower hemolytic activities (HI between 3000 and 4000) with a general principle that monodesmosides substituted at 3°C with glucose are more hemolytic than analogues substituted with glucuronic acid. Zanhic acid tridesmoside has an HI of 2000, and soyasapogenol-derived saponins show no activity.

### ANTIFUNGAL ACTIVITY

Fungitoxic activity can be of interest from both nutritional as well as pharmacological points of view. Highest sensitivity shows *T. viride*, and this fungus has been broadly used for alfalfa saponin quantitation. But even this fungus shows differential sensitivity to structurally divergent saponins. The most active, again, is medicagenic acid and its 3-O-glucoside. Their activity is 10 to 40 times higher than any other bidesmosidic saponins (Table 3). Zanhic acid and soyasapogenol glycosides do not show any activity.

The high fungitoxic activity of 3-O-glucosides suggested the idea of using this compound as the basis for developing a new group of antimicotic agent. It was shown that the compound displayed considerable activity against medically important yeasts, e.g., *Candida*, *Torulopsis*, and *Geotrichum* ssp. MICs obtained by both agar and broth dilution methods ranged from 3 to 15 µg/ml (Polacheck et al., 1986). Our efforts to support these findings failed. Experiments performed by Prof. A. Clark in the United States with our highly pure forms of several saponins, including 3-O-glucosides of medicagenic acid

TABLE 3. Comparison of some selected biological activities of individual alfalfa saponins.

Compound	Hemolytic Index	<i>T. viride</i> IA <sup>a</sup> <sub>50</sub>	Membrane Depolarization <sup>b</sup>	Mutagenicity <sup>c</sup>	Cytotoxicity <sup>d</sup>
MaNa <sup>+</sup>	11,896	1.7	1.77	none	na
3Glu Ma	18,157	1.6	2.45	none	none
3Glu,28Glu Ma	None	33.0	0.50	na	na
3Glu,28AraRhaXyl Ma	4,294	13.5	3.04	na	na
3GluA,28AraRhaXyl Ma	3,581	47.5	3.62	na	none
Soyasaponin I	none	none	none	none	na
Zanhic tridesmoside	2,000	none	6.22	na	none

<sup>a</sup> Saponin concentration (mg/100 ml of medium) resulting in 50% inhibition of fungus growth.<sup>b</sup> Total mV rat intestinal transmural potential difference fall in 10 minutes (Oleszek et al., 1994).<sup>c</sup> Ames test with *S. typhimurium* strains TA97, TA98, TA100, TA102, saponin concentration 200–500 µg/plate (Czeczot et al., 1994).<sup>d</sup> MTT assay, human colon cancer HT29 cells, saponin concentration 0–30 µg/ml (Lacaille-Dubois and Oleszek, unpublished).

using the agar-well diffusion assay at a concentration of compound of 1 mg/ml, showed no essential activity against *Candida albicans* B311. No such activity was observed for the mixture of alfalfa root saponins and for 3Glu28Glu medicagenate. Some fungi like *T. viride*, *Candida albicans*, *Aspergillus flavus*, *A. fumigatus*, *Saccharomyces cerevisiae*, *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Trychophyton mentagrophytes*, and *Mycobacterium intracellulare* did not show any sensitivity to zanhic acid tridesmoside when present in the broth at the concentration of 1 mg/ml (Oleszek and Jurzysta, 1992).

## MEMBRANE ACTIVITY

There is evidence that saponins can influence the digestion and absorption of other nutrients/pharmaceuticals by interacting with mucosal cell membranes, causing permeability changes or the loss of activity of membrane-bound enzymes. A study of the effect of saponins isolated from different plant sources on transmural potential difference in mammalian small intestine showed considerable variation in response to particular compounds. The saponin mixture from alfalfa tops, consisting predominantly of 3GluA28AraRhaXyl medicagenate (Oleszek, 1991), was the most potent depolarizer (Gee et al., 1989). This study showed that basic glycoalkaloids in potato and tomato and the complex bidesmosides from Gypsophila, Quillaja, and alfalfa are most potent, while soyasaponin shows only weak activity. However, maximum change in transmural potential difference for alfalfa saponins was shown in two studied saponin concentrations to be twice as much as for the others.

From evidence to date, it is clear that the chemical structures of a saponin play a significant part in determining the nature and extent of response of the gut (Lacaille-Dubois, 1996). Structurally divergent alfalfa saponins also showed such a dependence (Oleszek et al., 1994). It was clear that the structure of the aglycone is a predominant determinant of activity; medicagenic acid glycosides showed lower activity at 1 mM than zanhic acid glycosides at the concentration of 0.5 mM. But even between the saponins with the same aglycone, pronounced differences were recorded in total mV fall in 10 minutes. The lowest value (0.50) was obtained for 3Glu28Glu medicagenate, which was almost inactive. Monodesmosidic 3-O-glucoside medicagenate and medicagenic acid sodium salt showed higher activity (2.45 and 1.77, respectively). Bidesmosidic 3Glc28AraRhaXyl and 3GluA28AraRhaXyl medicagenates showed PD fall of 3.04 and 3.62 mV, respectively. At the same time, for zanhic acid tridesmoside at two times lower concentration (0.5 mM), PD fall was 6.22 mV. These data not fully fit the proposed mechanism of saponin membrane activity. According to the model of Seeman (1974), the hydrophobic moieties of the saponin molecules combine with the membrane cholesterol to form the perimeter of a stable, ring-shaped structure in the plane of the

membrane. If intestine membrane activity mechanism is based on cholesterol affinity, the same way as hemolysis or antifungal activity, the sequence of activity should be similar in all cases: monodesmosides>bidesmosides>tridesmosides (Oleszek, 1990, 1996). The opposite can be noticed for our results on intestine membrane activity: tridesmoside > bidesmosides > monodesmosides. This discrepancy cannot be simply explained, and more research on a larger representation of compounds is needed. Nevertheless, high activity of zanhic acid tridesmoside encouraged us to study all other characteristics of this compound. Lacaille-Dubois (1992) studied the possibility of using alfalfa saponins for increasing transport of cisplatin across the cell membrane to enhance its efficiency in human colon cancer treatment. Four alfalfa saponins were tested in the concentration range of 0 to 30 µg/ml with MTT assay on colon cancer HT29 cells. It was shown that none of the alfalfa saponins potentiated the cytotoxicity of cisplatin, but these samples did not show any cytotoxicity either (Table 3).

## TOXICITY OF ZANHIC ACID TRIDESMOSIDE FOR ANIMALS

To determine toxicity of zanhic acid tridesmoside, it was administered to the hamsters. Prior to the experiments, animals were not given any feed for several hours. Water solutions of different concentrations of zanhic acid tridesmoside were prepared so that 1 ml of it was used per 100 g of animal body weight. Samples were administered in one dose, straight to the animal's stomach with a stomach tube. Approximate LD<sub>50</sub> value for tridesmoside was calculated to be 562 mg/kg of body weight, and according to the scale of toxicity, the compound can be classified as moderately toxic (Oleszek et al., 1995). In comparison to the data published by Vogel and Marek (1962) for a number of plant saponins, their LD<sub>50</sub> values measured for rats ranged between 50 and 160 mg/kg. Chandel and Rastogi (1980) reported that LD<sub>50</sub> for ginseng saponins when fed to mice was 765 mg/kg, while hederagenin saponins from *Sapindus mukurosi* showed LD<sub>50</sub> of 1,625 mg/kg (Agrawal and Rastogi, 1974). Saponins from *Quillaia saponaria*, officially approved for use in food and drug industries, could be administered to rats at the dose of 400 mg/kg/day (Gaunt et al., 1974) without any toxic symptoms. Drake and co-workers (1982) claimed that, after some growth perturbances at the beginning of experiment, rats could be fed with 1,500 mg/kg for two years without any symptoms. Thus, zanhic acid tridesmoside from alfalfa seems to be more or less at the same level of toxicity as other triterpene saponins.

Some symptoms of bloating were observed in necropsy of hamsters; the intestines were heavily filled with gas. Because animals were not fed for several hours prior to the administration of saponins any other reason for this phenomenon apart from the influence of saponins can be reasonably excluded. Similar effects were reported by Lindahl and co-workers (1957), who observed

bloating syndromes in sheep administered pure saponins, and Klita and colleagues (1996), who showed that administration of 800 mg/kg of saponins in sheep induced pathological changes similar to those observed at bloating. These effects are not fully understood, but recent publications seem to exclude the role of alfalfa saponins as bloating agents (Majak et al., 1980; Hall and Majak, 1989).

## CONCLUSIONS

Our research findings indicate that early work on the nutritional/pharmaceutical application of alfalfa saponins must be revised due to the poor characterization of saponin preparations used. Biological tests used for saponin determination did not recognize the structural diversity of individual saponins and did not recognize all saponins present in plant material, e.g., soyasapogenol and zanthic acid glycosides.

Alfalfa can be a promising source of saponins for nutritional/pharmacological purposes. Plant material is easily available, and biological activities of these compounds are not much different than that of saponins obtained from other plant sources, e.g., Quillaia. Especially promising can be zanthic acid tridesmoside, as it is very soluble in water and shows quite low toxicity and high membrane affinity. For this purpose, alfalfa variety and seasonal changes in saponin concentration must be strictly determined prior to the appropriate plant material selection.

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# **Saw Palmetto: Critical Review, Chemistry, and Application**

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## **INTRODUCTION**

**T**HE prostate is a walnut-sized gland located just below the bladder. Benign prostatic hyperplasia (BPH) is a non-cancerous enlargement of the prostate gland. While the etiology of BPH is unknown, increasing age and the presence of androgens, such as testosterone and dihydrotestosterone, are thought to be the primary risk factors. The four conditions related to BPH include the following:

- (1) Anatomic prostatic hyperplasia
- (2) Prostatism
- (3) Urodynamic presence of obstruction
- (4) Response of the bladder muscle to obstruction

Patients with anatomic prostatic hyperplasia and urodynamic presence of obstruction are said to have "silent" obstruction, while the condition normally characterized as BPH also includes symptoms of prostatism and a response of the bladder muscle to obstruction (U.S. Department of Health and Human Services, 1994). The symptoms of prostatism and/or BPH can be characterized as irritative or obstructive. Irritative symptoms include increased frequency of urination, urinating during the night (nocturia), decreased voided volume, sensory urgency, and urgency incontinence. Obstructive symptoms include hesitancy, decreased stream, terminal dribbling, double voiding, and urinary retention (Stedman, 1990). After age 60, more than half of all men have BPH to some degree. The rate of BPH increases to 90% by age 85 (U.S. Department of Health and Human Services, 1994).

For general information on BPH and the use of saw palmetto in treatment of this condition, the reader is referred to several reviews (Bombardelli and Morazzoni, 1997; Buck, 1996; Carbin et al., 1990; Roylance et al., 1995; Wilt et al., 1998).

## TREATMENT STRATEGIES

The current conventional treatments for symptomatic BPH include watchful waiting, surgery, balloon dilation, and drug therapy, such as alpha blockers or finasteride (Proscar™, Merck Sharp & Dohme). Significant complications exist for surgery, including risk of infection and impotence. Nevertheless, transurethral resection of the prostate is second only to cataract surgery as the most common surgical procedure performed on the Medicare population. The cost is estimated to be in excess of two billion dollars per year. While balloon dilation results in fewer complications, it is less effective at relieving the symptoms associated with BPH. Alpha blockers relax the bladder neck and prostate smooth muscle, thus allowing ease of urination. While short-term efficacy has been proven in controlled trials, long-term efficacy is still unknown. Side effects associated with alpha blockers include orthostatic hypertension, dizziness, tiredness, and headache. Finasteride is a relatively new drug, approved by the Food and Drug Administration (FDA) in 1992. It is a 5 alpha-reductase inhibitor that blocks the conversion of testosterone to dihydrotestosterone. Finasteride has been shown to result in slight improvements in prostate size, peak urinary flow rate, and BPH symptoms with at least six months of treatment. Side effects of finasteride include decreased libido, ejaculatory dysfunction, and impotence (U.S. Department of Health and Human Services, 1994).

Because conventional treatments are associated with significant side effects, BPH patients are turning to some promising botanicals to help ease their symptoms. Table 1 lists the major botanicals used to help relieve the symptoms associated with BPH (Martindale, 1993).

Of the botanicals listed in Table 1, saw palmetto has the most research to support its use for symptoms associated with BPH. A member of the fan palms, saw palmetto grows in the South Central and South Eastern regions of the United States. The plant matures up to approximately 20 feet with leaf clusters attaining a length of two or more feet. The brownish-black berry is harvested commercially for use in the dietary supplement and pharmaceutical industry (Lawrence Review of Natural Products, 1994). Saw palmetto has been found to contain a variety of free fatty acids ranging from C6 to C20. Other components include beta sitosterol in low concentrations along with fatty alcohols, flavonoids, and terpenes. Reviews of the chemical composition of saw palmetto are available (Bombardelli and Morazzoni, 1997; Hatinguais,

TABLE 1. Common botanicals used to alleviate the symptoms associated with BPH.

Botanical—Amount/Day	Commercial Products		
Saw Palmetto ( <i>Serenoa repens</i> )—320 mg/day	Permixon forte Remigeron Prostagutt Curbicin	PA109 Prostagalen Prostaselect Prostavigol	Strogen
Beta Sitosterol—60 mg/day	Harzol		
Nettle ( <i>Urtica dioica</i> )—150–300 mg/day	Bazoton		
Pumpkin Seed ( <i>Cucurbita pepo</i> )—80 mg/day	IDS 23 Prostatonin Curbicin	Franufink Kurbis-Granulat Prostamed Uvirgan	
Pygeum ( <i>Pygeum africanum</i> )—100–200 mg/day	Cysto-Fink Prosta Fink N Prostatonin Prostamal		

et al., 1981; Jommi, et al., 1988; Lawrence Review of Natural Products, 1994; Neuzil and Cousse, 1993; Wajda-Dubos, et al., 1996).

## BPH CAN BE TREATED WITH EXTRACTS OF SAW PALMETTO

The benefits of saw palmetto for men with mild to moderate BPH are well-supported by the scientific literature. Lowe et al. (1998) and Wilt et al. (1998) have recently published meta-analyses of the saw palmetto clinical research. Lowe et al. (1998) included 13 studies (21–180 days in length) with 1961 men receiving saw palmetto. In these studies, saw palmetto was compared to placebo (7), to finasteride (1), to alfuzosin (1), to prazosin (1), and to pygeum and placebo (1). Data were also taken from two large open label studies. On average, it was reported that peak urinary flow was increased 1.87 ml/second over that seen with placebo ( $p < 0.001$ ). Also observed was a significant decrease in the number of nocturnal urinations. Wilt et al. (1998) looked at 18 randomized controlled trials including 2939 men with a mean study duration of nine weeks (4–48 weeks). Of these, treatment allocation was adequate in nine studies, while 16 were double-blinded. Compared to placebo, men receiving saw palmetto demonstrated significantly decreased urinary tract symptom scores, decreased nocturnal urination, improved self-rating of urinary tract symptoms, and increased peak urinary flow. Likewise, the effects of 320 mg/

day of saw palmetto were similar to those observed with Finasteride treatment, though with fewer side effects.

## THE ACTIVE COMPONENTS OF SAW PALMETTO ARE NOT FULLY ELUCIDATED

The active phytochemicals in saw palmetto have yet to be elucidated. In fact, it has been suggested that several phytochemicals may promote the beneficial effects observed with saw palmetto. Through *in vitro* research, various mechanisms of action have been proposed for sitosterols, which are found in saw palmetto. These include anti-inflammatory effects, alteration of cholesterol metabolism, direct inhibition of prostate growth, antiandrogenic or antiestrogenic effects, and a decrease in available sex hormone-binding globulin (Lowe and Ku, 1996). Several human clinical studies have supported the use of isolated sitosterols for symptoms associated with BPH. A recent study by Klippel et al. (1997) found that in 177 subjects with BPH, those consuming 130 mg of free beta sitosterol daily for six months demonstrated significant improvements in the international prostate symptom score (IPSS), quality of life index, peak urinary flow, and post-void residual urinary volume compared to those consuming placebo ( $p < 0.01$ ). Similar results were obtained from a six-month study with 200 subjects consuming 20 mg of beta sitosterol three times daily compared to placebo (Berges et al., 1995).

A fraction containing acid lipophilic compounds isolated from saw palmetto was found to inhibit the biosynthesis of cyclooxygenase and 5-lipoxygenase to a similar degree as the whole extract. Conversely, isolated fatty alcohols and sterols showed no inhibitory effect on these arachidonic acid pathway enzymes, suggesting that the anti-inflammatory activity of saw palmetto is due to the presence of acidic lipophilic compounds (Breu et al., 1992).

Saw palmetto extract has been shown to dose-dependently inhibit the 5 alpha-reductase enzyme in the prostate epithelium and stroma. The main fatty acids responsible for this effect appear to be lauric and myristic acid. The non-saponifiable subfraction consisting mainly of phytosterols also resulted in inhibitory effects, though to a lesser extent than that observed for the free fatty acids (Weisser et al., 1996). Several *in vitro* studies also suggest that saw palmetto interferes with androgen action within the prostate (Plosker and Brogden, 1996). On the other hand, *in vivo* studies have found no effect of saw palmetto on 5 alpha-reductase inhibition or androgen receptor binding (Rhodes et al., 1993; Strauch et al., 1994; Weisser et al., 1997).

From the information presented, it appears that saw palmetto is a botanical with proven efficacy for improving the symptoms associated with BPH. On the other hand, the exact mechanism of action and active compounds present are still an area of uncertainty. Of the phytochemicals investigated to date,

beta sitosterol may be responsible for the benefits associated with saw palmetto, though the quantity present in the botanical is much less than what was utilized in the clinical studies. Free fatty acids are another possibility, though work still needs to be done to determine the exact free fatty acids responsible. Other long chain molecules, such as aliphatic alcohols, have been implicated (Jommi et al., 1983). A more attainable hypothesis may be that saw palmetto works by multiple modes of action and multiple chemistries or an interaction of chemistries rather than by one specific mechanism and compound.

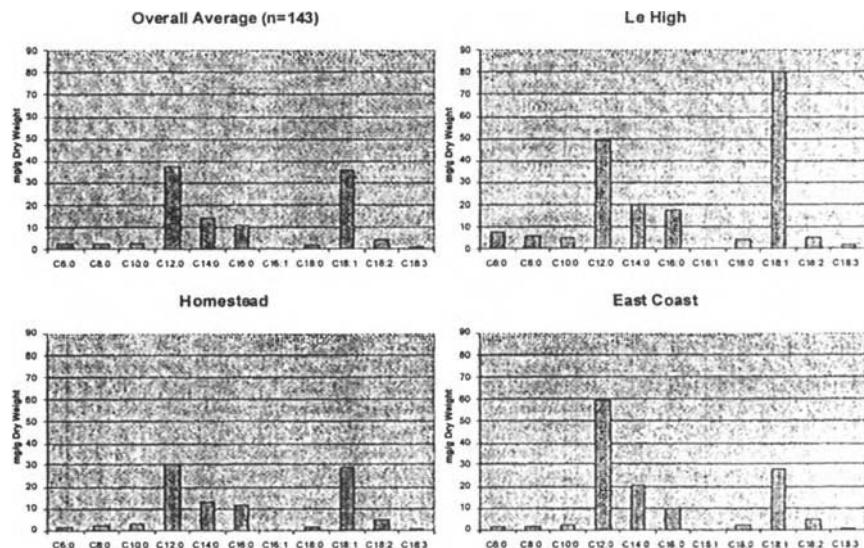
## CONTINUED PHYTOCHEMICAL RESEARCH ON SAW PALMETTO

The potential for multiple modes of action and multiple chemical species being involved in those actions demonstrates one of the prevailing difficulties in research with complex botanicals. In addition, the rather high placebo effect observed in BPH clinicals further confounds the search for active chemistries. One general approach to these problems is the attempt to find raw materials with sufficiently distinct chemistries and to test these for bioactivity. We have adopted this approach with saw palmetto using fatty acid analysis as a measure of the genetic variability of the species. Such an approach has been used in the variability analysis of other crops and for chemometric analysis (Garcia-Lopez et al., 1996; Pathak et al., 1994; Rojas et al., 1994).

We surveyed saw palmetto from more than eight million hectares, selecting 143 sites for analysis. Fatty acids were used as an indicator of potential variability. This was a convenient choice, as the majority of fatty acids (75–85%) occur in the non-esterified (free) state and they are implicated in the observed bioactivity of the preparation.

There appears to be a substantial degree of variation in the fatty acid profile of saw palmetto; some examples of variation are shown in Figure 1. The two major fatty acids, lauric and oleic, have been implicated in the mode of action of the extract (Weisser et al., 1996). They represent 40–70% of the total fatty acids and show the highest degree of variation. Myristic, linoleic, and linolenic acids, all of which have been implicated in bioactivity, also show some variation between sites. In contrast, the phytosterols, beta-sitosterol, campesterol, and stigmasterol, did not show much variation in content (not shown). Thus, careful selection of collection site can produce extracts with very similar sterol content, but widely varying fatty acid contents.

We also examined plant tissue differences in fatty acid composition. It was found that the preponderance of fatty acids are located in the fleshy pulp of the fruits (Table 2). If expressed on a weight per fruit basis, the differences become even more exaggerated (Table 2). It was interesting to note that phytosterol content between the two tissue types was the same. This again



**Figure 1** Variation in fatty acid profiles of saw palmetto berries in Florida: examples of geographic extremes.

offers the chance to evaluate different fatty acid compositions with constant sterols in a bioactivity-based assay.

In addition to fatty acids, carotenoids, tocopherols, and tocotrienols were also examined, as little information was available on these in the literature (Bombardelli and Morazzoni, 1997). Clear differences were not noted between

**TABLE 2.** Distribution and quantification of fatty acids in the pulp and seed of dried saw palmetto fruits.

Fatty Acid	Whole Fruit	mg/g Dry Weight		mg Total in Tissue <sup>a</sup>	
		Seed	Pulp	Total Seed	Total Pulp
C6:0	3.69 ± 0.85	1.05	4.77	0.52	3.77
C8:0	3.20 ± 0.41	3.09	2.77	1.51	2.19
C10:0	3.21 ± 0.28	4.92	1.67	2.41	1.32
C12:0	37.06 ± 4.44	21.45	43.31	10.51	34.22
C14:0	15.04 ± 1.75	4.25	21.11	2.08	16.68
C16:0	11.45 ± 1.16	5.02	14.93	2.46	11.80
C16:1	0.30 ± 0.08	0.01	0.46	0.01	0.36
C18:0	2.14 ± 0.30	2.24	1.79	1.10	1.41
C18:1	39.22 ± 4.27	17.14	48.69	8.40	38.47
C18:2	4.58 ± 0.45	7.64	1.79	3.74	1.41
C18:3	1.01 ± 0.10	0.07	1.61	0.03	1.27
C20:0	0.16 ± 0.03	0.07	0.17	0.03	0.13

<sup>a</sup> Weight of whole fruit = 1.26 ± 0.12 g; seed = 0.49 ± 0.05 g; pulp = 0.79 ± 0.11 g, n = 10.

TABLE 3. Distribution of carotenoids, tocopherols, and tocotrienols between tissues of dried fruits of saw palmetto.<sup>a,b</sup>

Carotenoids	$\mu\text{g/g}$ Dry Weight	
	Seed	Pulp
Lutein	n.d.	3.3
beta-Carotene	n.d.	121.2
cis/trans Phytoene	219.7	315.7
cis/trans Phytofluene	101.2	67.0
Tocopherols/Tocotrienols		
alpha-Tocotrienol	65.7	n.d.
gamma-Tocotrienol	23.9	n.d.
delta-Tocotrienol	18.9	n.d.
gamma-Tocopherol	n.d.	57.5
delta-Tocopherol	n.d.	26.9

<sup>a</sup> n = 10.

<sup>b</sup> n.d. limits of detection were less than 4.0  $\mu\text{g/g}$  dry weight.

different populations; most differences were directly related to the age of the fruit at harvest (not shown). However, differences were noted between plant tissue types (Table 3). There were clear tendencies to find colored carotenoids in the outer, pigmented tissue. We also found large amounts of the colorless carotenoids, phytoene and phytofluene, in both tissue types. In addition, we noted the tendency for tocopherols and tocotrienols to be tissue specific (Table 3). Although neither of these chemical classes are directly implicated in the control of BPH, their presence may contribute to the overall health of prostate tissue and may have modifying effects on other active components of the extract.

## CONCLUSION

The complexity of saw palmetto extracts mirrors its potential multiple modes of action in the control of BPH. In addition, varied plant chemistries are present that can further influence prostate health and may have an impact on the activity of phytochemicals more directly related to the action of saw palmetto on BPH. Continued exploration of the phytochemical profile, or genetic variation in the profiles, may contribute to the understanding of the mode of action of this botanical.

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# **Effect of Garlic on Serum Cholesterol Levels**

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## **INTRODUCTION**

**G**ARLIC has a long history of use worldwide for a variety of culinary and medicinal purposes, and many potential health benefits have been widely claimed. Some of these potential health benefits include cholesterol lowering, blood pressure lowering, enhanced immune function, decreased blood coagulation, and anti-oxidant properties. At this time, a fairly extensive body of scientific studies has been published in medical journals on the putative cholesterol-lowering benefits of garlic in particular. The studies conducted in this area fall into two broad categories: (1) mechanistic studies that assess the impact of various garlic compounds on cholesterol metabolism using cell or tissue cultures, or animal models, or (2) human clinical trials examining the serum cholesterol response to garlic intake administered as any of several different garlic formulations. While the mechanistic studies have consistently reported an effect of garlic compounds on cholesterol metabolism, the clinical trial evidence for a cholesterol-lowering effect in humans remains controversial with an almost equal number of trials reporting a significant effect as those reporting no detectable effect. In an effort to summarize current knowledge and understanding of the effect of garlic intake on serum cholesterol, these studies will be reviewed and critically evaluated.

## **GARLIC FORMULATIONS AND COMPOUNDS**

In an era when garlic consumption involves a multibillion dollar industry

worldwide (*Nutrition Business Journal*, 1998), there are a variety of different ways for an individual to ingest one form or another of the "stinking rose." Fresh, raw garlic can be minced, pressed, sauteed, baked, pickled, boiled, and juiced. The rapidly growing garlic supplement industry has developed processing methods for dehydrated garlic tablets, oil-macerated garlic, steam-distilled garlic oil, and aged-alcoholic garlic extract (solid or liquid). The putative health benefits of these various garlic preparations are associated with the sulfur-containing compounds found in these plants from the *Allium sativum* genus. However, it remains unclear exactly which garlic sulfur compound(s) is or are the primary bioactive agents. Eight different thiosulfinates that are thought to possess biological activity have been identified. The major thiosulfinate, allicin, accounts for approximately half of the total thiosulfinates (Calvey et al. 1997). It is also known that most of the organosulfur compounds found in garlic are derived from allicin (Freeman and Kodera 1995). Allicin is generated from alliin by the enzyme alliinase, which is released when garlic is physically disrupted. However, allicin is an unstable and volatile molecule that has a very short half-life in the human body—it is quickly converted to other transformation products that are believed to be the ultimate active bio-agents (e.g., S-alkenyl-cysteine sulfoxides,  $\gamma$ -glutamyl S-alkenyl cysteines, S-allyl-cysteine, and S-allyl-mercaptocysteine (Lawson and Wang 1993). Adding to this complexity is the fact that the thiosulfinate content of garlic products can vary widely due to differences in cultivation, harvesting, processing, and by geographical region (Block et al. 1992). Therefore, given the tremendous interest in the potential health benefits of garlic, it is important to recognize the uncertainty that remains as to

- (1) What is the best delivery vehicle (e.g., raw cloves vs. dried vs. oil)
- (2) What is/are the most bioactive sulfur-containing compound(s) (e.g., allicin vs. S-allyl cysteine vs. possible synergistic effects)
- (3) Whether these might differ for different health outcomes (e.g., cholesterol lowering vs. blood pressure lowering).

## MECHANISTIC AND ANIMAL STUDIES

Several mechanistic studies have consistently reported an inhibitory effect of garlic on cholesterol biosynthesis. Gephardt and Beck (1996) used rat hepatocytes to test the effect of allicin, diallyl disulfide, allyl mercaptan, and vinyl dithiins on both early and late steps in the metabolic pathway of cholesterol biosynthesis. Allicin proved to be the most effective inhibitor, but the other sulfur-containing compounds also inhibited biosynthesis to varying degrees, at varying points in the metabolic pathway. Yeh and Yeh (1994) observed that three different fractions of garlic extracts—petroleum ether, metha-

nol, and water extracts—all showed decreased rates of [<sup>1-14</sup>C]acetate incorporation into cholesterol (37–64%), suggesting inhibition of synthesis. Gephhardt (1993) examined the effect of water-soluble garlic extracts on several different enzymatic steps in the biosynthetic pathway. He observed that alliin was without effect, but when converted to allicin, there was inhibition in the late steps of cholesterolgenesis. In another study (Sendl et al., 1992), a modified liver homogenate model was used to assay for the inhibition of cholesterol biosynthesis. It was reported that each of five individual sulfur-containing compounds—ajoene, methylajoene, allicin, 2-vinyl-4H-1,3-dithiin, and dialydisulfide—inhibited cholesterol synthesis by 37 to 72%.

The concentration of cholesterol in the serum can be lowered either by decreasing the synthesis and secretion of cholesterol, as suggested above, or by increasing clearance. Regarding clearance, one group of investigators has reported that lyophilized garlic feeding enhanced excretion of the neutral and acidic steroids in rats fed cholesterol or lard. Serum cholesterol concentrations were decreased by 30% (Chi et al. 1982).

In support of the mechanistic evidence cited above for a plausible hypocholesterolemic effect of garlic intake, several animal studies have reported a serum cholesterol-lowering effect. Bordia et al. (1977) reported that garlic oil fed to rabbits (amount of oil proportional to 1 g raw garlic/kg body weight) lowered serum cholesterol and triglyceride levels and reduced aortic atheroma by approximately one-half in a model of experimentally induced atherosclerosis. In 1980, Dixit and co-workers observed that garlic powder given to dogs and Presbytis monkeys at 4.25 mg/kg body weight was more effective in decreasing serum cholesterol and triglyceride levels than gugulipid (a plant substance with potential cholesterol-lowering effects). In another controlled study, chickens fed garlic powder demonstrated altered lipid and cholesterol metabolism. The supplementation caused a reduction in plasma, liver, and muscle cholesterol. HMG CoA reductase and alpha hydroxylase activity were significantly reduced (Konjufca et al. 1997). Given the substantial body of mechanistic and animal evidence suggesting a hypocholesterolemic effect of garlic, a large number of human clinical trials have now been conducted.

## CLINICAL TRIALS

Extensive clinical trial data have been reported on the effect of garlic intake on serum lipids. In 1993 and 1994, two meta-analyses reported that daily intake of garlic, primarily in the form of garlic supplements, in doses equivalent to approximately one clove (~800–1000 mg dried powdered garlic), reduced serum cholesterol levels by 9% (Warshafsky et al. 1993) or 12% (Silagy and Neil 1994) in adults with elevated cholesterol levels. However, both of these groups of investigators expressed concerns with the scientific merit of the

published trials that were included in their meta-analyses. Warshafsky identified 28 clinical trials, but excluded 23 of the trials from the final analyses due to low scientific merit, inclusion of participants with normal cholesterol, or for providing insufficient data for the calculation of effect sizes for the meta-analysis. Based on the remaining five trials whose results were pooled, the following statement was made in the discussion: "Although our data support the claim that oral garlic therapy decreases serum cholesterol levels in persons with increased levels, the quality of the included studies was not optimal" (Warshafsky et al., 1993, p. 603). This makes any conclusions suspect. Similar findings and caveats were reported a year later by Silagy and Neil (1994). They identified 25 randomized, controlled trials and excluded 11 of these because of low scientific merit, short duration, or insufficient data for calculating effect sizes. The results of the remaining 14 studies were presented separately in two categories—"non-powder preparations" and "powder preparations"—and a significant cholesterol-lowering effect was reported for each of these sets of pooled studies. However, this conclusion was also tempered with qualifying statements: ". . . more rigorously designed and analyzed trials are needed. . . . The quality assessment of the trials was generally poor." The concerns and caveats reported in these two meta-analyses were well justified and suggested that further clinical trials were warranted.

Between 1993 and 1999, at least 11 additional clinical trials have been published that tested the effect of garlic intake on serum lipid levels. Of these, six reported a significant benefit from garlic intake (Jain et al. 1993; Steiner et al. 1996; Adler and Holub 1997; Bordia et al. 1998; Morcos, 1997; Lash et al. 1998), while five reported no detectable benefit (Simons et al., 1995; Neil et al. 1996; Isaacsohn et al., 1998; Berthold et al. 1998; Gardner, submitted for publication). Because a simple vote count in this case will obviously not resolve this controversy, a closer look at the design and scientific merit of these individual studies is warranted. In order to critically evaluate these data, emphasis will be given to the flaws or limitations in specific design, conduct, and analyses of studies that were identified in the clinical trials published before 1993. These flaws or limitations included recruitment methods, sample size, study duration, control and documentation of potential confounders (weight, diet, exercise), compliance rates, use of standardized laboratory methods for lipid assessment, documentation of side effects and drop outs, lack of standardization of garlic compounds, and potential conflicts of interest.

## STUDIES REPORTING A BENEFICIAL LIPID RESPONSE TO GARLIC SUPPLEMENTATION

In 1993, Jain et al. reported results from a placebo-controlled study that randomized 42 adults with total cholesterol levels greater than or equal to 220 mg/dL, to a 12-week intervention, using a parallel design. Garlic tablets

containing 900 mg/day of dried garlic powder, standardized (to 1.3% allicin?—not reported, assumed from similar trials), were provided as three daily doses of 300 mg/dose. LDL-cholesterol levels in the garlic group were 188, 172, and 168 mg/dL at baseline, six weeks, and 12 weeks, respectively. LDL-cholesterol levels for the placebo group averaged 191, 180, and 185 at the corresponding timepoints. The differences between garlic and placebo were significant at only the 12-week point ( $p<0.05$ ). Results for total cholesterol levels were similar, and there were no significant differences in HDL-cholesterol or triglycerides. Lipid assessments were performed using standard enzymatic methods in a laboratory participating in the Centers for Disease Control (CDC) standardization program. Body weight was monitored and remained stable throughout the 12 weeks for both groups, and side effects were reported but apparently did not affect the conduct of the study. No data are presented to document diet or exercise habits during the study. Compliance and drop-outs were also not reported. Funding for the study was provided by the supplier of the garlic tablets, and so the potential for a conflict of interest exists. The design of the study appeared adequate, but the reporting and apparent conduct of the study were questionable.

One of the studies reviewed here used an aged, garlic extract for an intervention (Steiner et al., 1996). This double-blind, cross-over study recruited men with serum cholesterol levels between 220 and 290 mg/dL. Other than being hypercholesterolemic, the participants were not characterized, and a description of the recruitment methods was not provided. After four weeks, during which participants were advised to follow a National Cholesterol Education Program Step I diet, 52 men were randomly assigned to either garlic capsules containing 800 mg/day of aged, garlic extract powder, or a starch and cellulose-based placebo. The regimen of capsules involved nine capsules daily, taken in sets of three, with meals. After six months of either garlic or placebo, the men then received the opposite treatment in cross-over fashion for four months. Serum lipids were assessed once each month throughout the study using enzymatic methods; no mention was made whether the lab doing the analyses participated in a lipid standardization program. The LDL-cholesterol levels were significantly lower with the garlic than with the placebo ( $p = 0.004$ ), but the magnitude of the maximal differences was only 4.6%, or roughly 7 mg/dL. Changes in total cholesterol results paralleled the LDL-C results, and there were no significant differences for HDL-C or triglycerides. Besides the inadequate characterization of the study population described above, this clinical trial appears problematic in several areas. The authors of the study provide no justification for the difference of six months for the first phase of the cross-over and four months for the second phase, although they do report that the maximal effects appeared to be reached by three months. After four weeks of run-in with advice to follow NCEP Step I guidelines, the randomization generated two groups with what appear to be substantially different LDL-

cholesterol levels, roughly 160 vs. 145 mg/dL in the garlic vs. placebo groups, respectively. This differential would favor a greater reduction of LDL-cholesterol while on the garlic treatment for the group that began with garlic in the first phase of the cross-over (i.e., attributable to regression to the mean), a possibility that is not discussed. Body weights were reported to remain stable throughout the study, but there is no documentation of diet or exercise habits as potential confounders. The blinding of the study materials proved ineffective with 70% of those on garlic correctly identifying their assignment, and compliance was reportedly worse with the garlic capsules than with placebo. The drop-out rate was substantial, with 11 of 52, or 21%, not completing the study, limiting the generalizability of the findings. In general, the data were difficult to interpret, and excessive statistical testing was done, creating a problem with proliferation of Type I errors (i.e., multiple testing increases the likelihood of generating findings that appear to be statistically significant but are in fact due to chance alone). The concerns raised here suggest that these data, contrary to the authors' stated conclusion, do not provide strong support for a cholesterol-lowering effect of aged garlic extract at a level that merits clinical relevance.

A well-controlled test of garlic vs. placebo was conducted by Adler and Holub (1997) and reported a net decrease of 13% in LDL-cholesterol using dried-powdered Kwai® (Lichtwer Pharmaceuticals) garlic tablet supplementation at a dose of 900 mg/day for 12 weeks. In this investigation, 50 men with serum cholesterol levels greater than 200 mg/dL were randomized to one of four groups: placebo ( $n = 12$ ), garlic ( $n = 13$ ), fish-oil ( $n = 12$ ), or garlic + fish oil ( $n = 13$ ). Four drop-outs during the study were distributed across three of the four groups. For the purpose of this discussion, only the placebo and garlic arms of the study will be considered. Weight and diet were monitored during the trial and were reported to be similar in all treatment arms. Blinding was reported to be relatively ineffective—the majority of participants guessed their treatment assignment—but compliance was >80% in all 46 participants who completed the study. Serum lipids were assessed every three weeks using standard enzymatic methods. No mention was made whether the lab doing the lipid analyses participated in a lipid standardization program, and it was not reported whether the Kwai® garlic tablets were standardized to allicin content, as they were in other trials. No apparent conflict of interest exists in this trial because funding came from a source other than the manufacturer of the garlic tablets. Despite the limitations noted above, on the whole, the study was well controlled and documented. These findings lend credible support to the hypocholesterolemic effect of garlic supplementation. However, due to the small sample sizes in each treatment arm, the conclusions should be interpreted cautiously.

Three other published trials reported a cholesterol-lowering effect of garlic in 1997 and 1998, but each of these was of relatively low scientific merit.

Bordia et al. (1998) reported a 13% reduction in total cholesterol with garlic oil supplementation for three months relative to placebo. However, the control and reporting of weight, diet, exercise, compliance, side effects and drop-outs were either insufficient or absent. Morcos et al. (1997) reported a 10% reduction in LDL-C levels among adults receiving a combination of fish oil and garlic relative to placebo, but in this trial, unlike the Adler and Holub study (1997), there was no treatment arm that used garlic alone (i.e., all participants who received garlic also received fish oil). A brief report by Lash et al. (1998) reported a cholesterol-lowering effect of garlic supplementation in renal transplant patients relative to placebo; however, similar to the study by Bordia and co-workers (1998), there was little documentation of the conduct of the study, and the funding source of the study involved a potential conflict of interest.

Taken as a whole, these six clinical trials have done little to address earlier criticisms of a lack of rigor and scientific merit in clinical trials examining the lipid-lowering effects of garlic intake. With the exception of the Adler and Holub study (1997), these clinical trials fail to provide compelling evidence that garlic supplementation, as administered, is efficacious in lowering serum cholesterol levels in moderately hypercholesterolemic adults.

## STUDIES REPORTING AN INSIGNIFICANT LIPID RESPONSE TO GARLIC SUPPLEMENTATION

Simons et al. (1995) conducted a double-blind, placebo-controlled, randomized trial investigating the effect of garlic supplementation on serum lipids among mild to moderate hypercholesterolemic subjects. The cross-over design of this study included 12-week treatment periods as well as four-week run-in and wash-out periods. Similar to other studies, the garlic formulation used was Kwai® dried, powdered garlic tablets at a dose of 900 mg/day, with a standardized allicin content of 1.3% by weight. Recruitment and baseline characteristics of the 31 participants enrolled were clearly documented. Weights and daily nutrient intakes were controlled and reported, compliance was high (97%), side effects were noted but apparently did not interfere with the study, and there were only two drop-outs from the original sample of 31. Plasma lipid profiles were virtually identical for the garlic and placebo phases of the trial with no significant differences. The trial was funded by the manufacturer of the garlic formulation used in the trial, but, in this case, the possibility of a conflict of interest works in reverse and perhaps lends additional credibility to the findings, because the authors reported finding no demonstrable effect of garlic ingestion on lipids and lipoproteins.

In 1996, the authors of the 1994 meta-analysis reported the findings from their own clinical trial (Neil et al. 1996). In the largest of any of the trials to be reviewed here, 115 hypercholesterolemic patients were randomized to

receive either Kwai® dried, powdered garlic tablets (900 mg/day, 3 × 300 mg/tablet, standardized to 1.3% alliin) or placebo for six months in a parallel design. Of the 115 randomized, 106 completed the trial (<10% drop-outs). The participant recruitment and relevant baseline characteristics at the study's onset were well documented. However, the control over the conduct of the study for the six-month intervention phase was problematic. As reported in the methods, the six-month intervention phase involved only minimal follow-up, with clinic visits at baseline, two months, and end of study. The potential confounding from lifestyle factors, such as diet and exercise, was not addressed. Compliance was poor, with roughly half the participants taking less than 75% of their assigned garlic or placebo tablets. Plasma lipid levels were not significantly different in the two treatment arms at six months. The absence of a difference in lipid levels could be due to a true lack of physiological effect, but, in this study, it could also have been due, at least partially, to poor compliance and confounding lifestyle variables. The potential for a conflict of interest exists because the study was partially funded by Lichtwer Pharmaceuticals, the manufacturer of the study's garlic formulation. But as with Simons et al. (1995), this would only add credibility to the findings, because the findings reported were for a non-significant effect.

A very recent and well-controlled study was reported by Isaacsohn et al. (1998). They conducted a randomized, placebo-controlled, garlic intervention study with a parallel design and a 12-week treatment duration. In this study, 50 hypercholesterolemic men and women, with a mean ± SD LDL-cholesterol of  $172 \pm 14$  mg/dL, were assigned to 900 mg dried, powdered garlic (Kwai®) per day, or placebo. Diet records analyzed at four, eight, and 12 weeks indicated no treatment group differences in nutrient intake, and weight remained stable and similar for the two groups. Compliance was estimated by pill count to be approximately 90%. The laboratory conducting the cholesterol analyses was CDC standardized and used standard enzymatic methods. Minimal side effects were reported, and there were a total of eight drop-outs in the study, four in each group. No significant differences were reported for LDL-C or any of the plasma lipids measured. The study was funded by the sponsor, Licher Pharma, and so again the potential for a conflict of interest appears to be negligible due to the conclusion of no effect.

Another recent trial was reported in 1998 and was again rigorously conducted, but in this case, the garlic formulation differed substantially from other studies. Berthold et al. (1998) used a steam-distilled garlic oil to test the effect on serum lipids. In this randomized clinical trial, 25 adults with elevated LDL-C (mean = 207 mg/dL) participated in a cross-over trial, with each phase of the cross-over being 12 weeks in duration. The dosage used was 10 mg/day of the steam-distilled garlic oil, which was reported to be roughly equivalent to twice the garlic dose of the dried, powdered formulations used in other studies, with an allicin content of 4000 units of allicin equivalents.

Compliance was high, averaging 98%. On-study diets were assessed using seven-day food records during each of the two treatment phases and were found not to differ. Standard enzymatic cholesterol methods were used in this study, although no laboratory standardization program participation was mentioned. Only one participant was excluded in the data analysis, and this involved only a missing fecal sample for additional analyses. Minimal side effects were reported, and none was serious enough to influence the study outcome. The funding source did not involve a conflict of interest. This investigation did not detect any significant lipid changes in the two treatment phases and concluded that garlic was ineffective in lowering elevated cholesterol levels in hypercholesterolemic adults. As mentioned above, although this was a rigorously controlled trial, the garlic formulation was substantially different from the dried, powdered formulations used in other studies and, therefore, should not simply be lumped together with the others when making overall conclusions. Of additional interest was the examination of biochemical markers for cholesterol metabolism in this study. Parallel to the absence of change in lipoproteins, no significant differences between the two groups were found in cholesterol absorption or synthesis.

Adding one more finding of a non-significant effect to those summarized above, our own research group has recently completed another trial in this area (Gardner et al., submitted for publication). In this study, 53 hypercholesterolemic (LDL-C 130-190 mg/dL), but otherwise healthy, men and women were recruited from the general population through newspaper and university campus advertising. The 12-week duration of this parallel design included three treatment arms. Participants were randomized to three tablets/day of either placebo (0 mg/day), half dose (500 mg/day), or full-dose (1000 mg/day) of a commercial, dried, powdered garlic tablet. Blood sampling was conducted at two-week intervals. Weight, diet, and exercise were monitored and remained stable over the 12 weeks for each of the three treatment arms. Compliance was determined by pill count to be 85%. Standardized laboratory methods were used for lipid assessments that were done in a CDC standardized laboratory. Minimal side effects were reported, and only two of the original 53 participants dropped out of the study, both in the first week due to scheduling conflicts. A limitation was that the tablets were not standardized in this study. The findings indicated that the full dose group ( $n = 16$ ) experienced an average 6% decrease in LDL-cholesterol relative to placebo ( $n = 18$ ), which was not statistically significant. The findings were similar for total cholesterol. The half-dose group ( $n = 17$ ) lipid profile was indistinguishable from the placebo. Funding was provided by the supplier of the garlic tablets, and, so again, given the non-significant findings, no conflict of interest is apparent.

In contrast to the six clinical trials reviewed earlier that reported significant effects, these five clinical trials reporting no significant effects were consistently more rigorous in design and more thorough in documenting compliance,

drop-outs, confounders, and side effects. With the exception of the study by Berthold and co-workers (1998), these recent trials reporting no significant effect of garlic all used dried, powdered garlic formulations. Therefore, a reasonable interpretation of these studies is that this particular garlic formulation has a negligible effect on serum lipid levels. These findings do not answer questions pertaining to other doses, other formulations, or other health outcome effects of the same garlic formulation.

## DISCUSSION AND CONCLUSIONS

Mechanistic studies have consistently reported significant effects of garlic compounds on cholesterol metabolism in various cell, tissue-culture, or animal models. But clinical trials using widely available forms of garlic supplementation, primarily dried, powdered garlic formulations, have failed to demonstrate a serum cholesterol-lowering effect in hypercholesterolemic adults of a magnitude that would be considered clinically relevant. In the large number of published clinical trials in this area reported in the last two decades, considerable heterogeneity in scientific merit is evident. Focusing in particular on the more recent trials reported from 1993–1999, the findings are highly inconsistent, with roughly half the trials reporting a significant cholesterol-lowering effect, and half reporting no significant effect. However, a critical examination of these studies suggests that, among the trials conducted most rigorously and documented most thoroughly, the effect of garlic supplementation on serum cholesterol is negligible.

There are several possible explanations for the apparent discrepancies between mechanistic studies and clinical trial results. One of these would be dosage. The majority of clinical trials have all used a fairly uniform dose of garlic, ~900 mg dried, powdered garlic extract/day, equivalent to approximately 1 to 1.5 cloves of fresh garlic per day. It would certainly be reasonable to experiment with larger doses, and it is perhaps somewhat surprising that this has not yet been pursued more thoroughly. It could be that, in the clinical trials, the concentrations of active garlic compounds reaching cholesterologenic tissues, such as the liver, are substantially lower than the concentrations achieved in mechanistic studies because garlic compounds are incubated directly with cells or tissues *in vitro*. Similar to the issue of dosage is compliance. Because compliance is typically less than 100% in clinical trials, but is virtually 100% for all studies in cells, tissues, or animal models, this would suggest that the opportunity to detect significant garlic effects is greater in the latter studies. Another possible explanation is that the active garlic compounds found to be effective in mechanistic studies may become inactivated in the human body during the process of digestion, absorption, circulation, and metabolism (suggesting the possibility of an important role for enteric-coated garlic tab-

lets). Along these lines, Lawson (1998) has recently suggested that the allicin yield of some dried, powdered garlic tablets may be low due to the inactivation of alliinase by gastric acid. He explains that the weak or absent effect of these supplements on serum cholesterol could be due to a low "effective allicin yield," which could be improved by altering the tablet formulation or by consuming crushed garlic cloves. Yet another possible explanation could be related to the fact that cholesterol biosynthesis is just one component of cholesterol homeostasis. Serum cholesterol concentrations are dependent on both input of cholesterol to the system (synthesis and secretion) and the clearance of cholesterol (receptor and non-receptor mediated fecal steroids and bile acids). If the rate-limiting step in this process for some hypercholesterolemics is largely clearance rather than synthesis of cholesterol, then the focus of the mechanistic studies on cholesterol synthesis may hold little relevance for predicting serum concentrations of cholesterol. These are only several possible explanations for the apparent discrepancies between the mechanistic data and the clinical trials of garlic and serum cholesterol; there may be others. However, no matter what the correct explanation may be, the recent clinical trial evidence fails to support a clinically relevant effect of garlic supplementation, in the doses and formulations described above, on serum cholesterol levels in hypercholesterolemic adults.

A brief mention of studies that have looked beyond serum lipid levels and metabolism to examine other potential mechanisms for anti-atherosclerotic effects of garlic is warranted. Two such studies were reported by Orekhov and Tertov (1997) and Orekhov and co-workers (1995). During a 24-hour incubation period, using serum and smooth muscle cells from patients with angiographically documented atherosclerosis, it was demonstrated that a garlic powder extract (GPE) significantly reduced the level of free cholesterol and cholesterol esters in the serum and also inhibited cholesterol accumulation and proliferative activity in the smooth muscle cells. Similarly, *ex vivo*, it was demonstrated that GPE caused a reduction in the amount of cholesterol accumulated in the cultured cells (Orekhov et al. 1995). These researchers later conducted a study to evaluate a mechanistic basis for GPE in reducing lipid accumulation in plaques by using atherosclerotic and normal intimal aortic cells exposed to atherogenic serum. As in the previous study, the GPE did reduce the lipid accumulation in these cells. It was also demonstrated that GPE inhibited acyl-CoA: cholesterol acyltransferase (involved in cholesterol ester formation) and stimulated cholesterol ester hydrolase (degradation of cholesterol esters), thus, providing a mechanistic basis for GPE in the prevention of lipid accumulation on human aortic cells (Orekhov and Tertov 1997).

Another recent study examined the impact of Kyolic® aged garlic extract (Wakankaga) on atherosclerosis in rabbits (Efendi et al., 1997). The rabbits were deendothelialized and were randomized to one of four groups, which included two standard diets, one with Kyolic® (I) and one without Kyolic®

(II); and two cholesterol supplemented diets, one with Kyolic® (III) and one without Kyolic® (IV). The dose of Kyolic® was 800 ml/kg body weight/day. Neither of the standard diet groups exhibited increases in cholesterol or atherosclerotic measures. The cholesterol-rich diets caused significant increases in cholesterol in both groups III and IV. Unexpectedly, cholesterol levels were not significantly lower in the Kyolic® group (IV) compared to group III. However, despite a lack of significant cholesterol lowering in the cholesterol-fed rabbits with Kyolic® (IV), they had significantly less fatty streak development, less cholesterol accumulation in the vessel wall, and less development of fibro fatty plaques compared to cholesterol-fed rabbits without Kyolic®. These findings suggest that Kyolic® provides protection against atherosclerosis that was not associated with cholesterol lowering in the blood, but may be due to reduced cholesterol uptake into the plaques.

## IMPLICATIONS

The implications of this review are restricted specifically to the types and doses of garlic supplementation commonly available at this time and their effect on serum cholesterol—these appear to be clinically ineffective. Many other aspects of garlic intake and health promotion continue to merit strong interest. It may be that these same, available, garlic supplements are effective at lowering blood pressure, enhancing immune function, improving coagulation factors, and/or increasing anti-oxidant capacity; however, these topics were beyond the scope of the current review. It may also be that larger doses of garlic supplementation or the design and production of different vehicles of garlic administration (e.g., enteric-coated tablets or garlic oil preparations) prove to be effectively hypocholesterolemic. Finally, an issue greatly relevant to human dietary practices is that garlic intake could have an indirect effect on serum cholesterol levels or other health outcomes. In the case of individuals who consume substantial quantities of garlic, some tend to consume specific foods with garlic (e.g., increased saturated fat intake due to the combination of garlic and butter on “garlic bread” or increased vegetable and fiber intake related to the flavoring of vegetable and grain dishes with garlic) that may themselves have an impact on serum cholesterol. Whether or not garlic itself is directly or mechanistically linked to health, increased fresh garlic consumption may be indicative of particular dietary patterns or habits that have a direct and causal health impact. In conclusion, the current clinical trial data suggest that commonly available, commercially prepared garlic supplements have a marginal or negligible effect on serum lipids. Further investigation of fresh garlic and enterically coated garlic supplements is warranted and should include careful identification and standardization of the various sulfur-containing garlic compounds.

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## Bioactives in Rice Bran and Rice Bran Oil

RUKMINI CHERUVANKY

### INTRODUCTION

**T**HREE is growing scientific evidence on the role of functional foods in the prevention and treatment of at least four major causes of death all over the world, namely, cancer, cardiovascular diseases, diabetes, and hypertension (Goldberg, 1994). Functional foods are defined as "modified natural foods or food ingredients which may provide health benefits, beyond the nutrients it contains" (Murthy, 1997). Functional foods are typically rich in phytochemicals, which are derived from natural plant products. These phytochemicals may have nutritive value or may be non-nutritive component(s) of foods. Phytochemicals that elicit biological activity are termed "bioactives." Bioactives eliciting medical and health benefits including prevention and treatment of diseases (Eisenberg et al., 1993) are termed "nutraceuticals." The marketing of nutraceutical products should be supported by scientific evidence and clinical data. It has been recognized on the basis of epidemiological evidence correlating diet and disease that phytochemicals aid the body in maintaining health and combating disease (Childs, 1997). Health authorities recommend that diets rich in whole grains, fruits, and vegetables are sources of phytochemicals and help in disease prevention (DeFelice, 1998). In the East, Asian countries have a long tradition of recognizing foods as medicines or as having unique health-promoting properties. As a tradition, the Japanese have taken a lead to market functional foods.

The food industry is responding to consumer demands for a healthier food supply by developing more nutrient-rich products as per U.S. Dietary Guidelines for Americans (Bloch et al., 1997). Food labeling provides reliable

information to consumers according to the Nutrition Labeling and Education Act (1990). The product nutrition panel may use Food and Drug Administration (FDA) pre-approved health claims based on sound scientific evidences. An increasing awareness of the potential benefits of functional foods and phytochemicals in the prevention and treatment of diseases is recognized. There is also a need to further investigate these foods for potential health benefits. The Foundation for Innovation in Medicine (FIM) has consistently maintained the position that medical and health claims that apply to food companies should be similar to those for dietary supplements and medical foods. Food companies are advised to conduct extensive research and development studies on their products (Murthy, 1997).

Rice and products derived from it offer a food source with a unique blend of bioactives that have significant health implications. The following review presents the chemistry, functions, and health benefits of rice products, based on numerous, well-documented scientific evidences.

## **DEFINING RICE BRAN**

Rice bran is a by-product of the rice milling industry. It is the portion of paddy between the hull and the white rice grain. The modern two-step rice milling process includes the removal of the hull, a component of little value, used in industries primarily in energy cogeneration. The second step is polishing of the kernel. The process of polishing removes the bran and germ of the kernel. When paddy is milled, brown rice (80%) is obtained. Brown rice is polished several times to get white rice, and the polishes put together, known as rice bran, account for 8% of white rice. During the process of polishing brown rice, phytochemically rich rice germ also gets mixed into the bran and provides essential nutrients.

The interest in rice bran is not only due to the dense nutrient and phytochemical content, but also to its enormous availability each year as reported by the Food and Agricultural Organization (FAO). Annual world production of rice is 543 million metric tons, and U.S. production of rice is 7 million metric tons (FAO, 1998). The U.S. availability of rice bran is 621,000 metric tons, which is primarily used as cheap animal feed. Although rice bran is rich in nutrients, it is underutilized due to its inherent instability. Rice bran will become rancid within a few hours of milling and, as such, is not suitable for human consumption. The shelf life of raw bran is less than a week. It is apparent that the stability and shelf life of rice bran is one of the primary factors in establishing the value of the product. If rice bran is properly stabilized, after milling, there are many possible applications in the food industry, and the potential utilization of the product as a dietary source of bioactives increases (Saunders, 1986).

There have been many processes developed to stabilize rice bran with little success. Several methods of stabilization have been developed over the years, but the methods developed provided a product that did not have sufficient stability to be considered as a viable food ingredient (Pillayar, 1978). The processes developed were not only ineffective, but also significantly reduced many of the bioactive compounds present in rice bran.

## **THE NEED FOR STABILIZATION**

Freshly milled rice bran has only 2.0% free fatty acids, which rapidly increases 5 to 6% at room temperature within 24 hours, and within a week, the raw rice bran loses its sensory qualities and becomes rancid. This makes it unfit for utilization as food/feed. This is because of the strong lipases and lipoxygenases present in the bran. The primary need for stabilizing the raw bran is to inactivate the lipases and lipoxygenases and to reduce bacterial load. The process of stabilization results in preserving the bioactives and maintaining better shelf life for better utilization of the product as a food or feed.

## **CRITERIA FOR STABILIZATION OF RICE BRAN**

The stability of rice bran is affected by the following three parameters: (1) hydrolytic stability, (2) oxidative stability, and (3) microbiological stability. Rice bran is rich in lipases, a group of hydrolytic enzymes that, on milling, come into contact with the fat in the bran, hydrolyzing the glycerides of the fat into free fatty acids and glycerol. The rapid increase in free fatty acids causes the bran to become organoleptically unacceptable in a short period of time. Free fatty acids are the yardstick to measure the hydrolytic stability of a product.

Rice bran is rich in fat (20–22%). In the presence of lipoxygenases present in the rice bran, the unsaturated fatty acids become oxidized at the double bond, generating free radicals. The free radicals generated continue the chain reaction, producing peroxides, superoxides, thiobarbituric acid reactive substances (TBARS), and the carbonyl end product, known as hexanal, within a short time. Hexanal production correlates very well with the rancid smell and sensory qualities of the product. Hexanal content is a measure of oxidative stability of a product. Lipoxygenase activity is highest in the germ fraction. This results in the poor stability and short shelf life of the product with loss of sensory properties as well as nutrients. The poor oxidative stability of the bran renders it unfit for utilization.

Microbial load also produces high free fatty acids. The product with high microbial load is unhygienic and is not safe for utilization as a health food or as an animal feed. Hence, stabilization of bran that reduces microbial load is imperative for its shelf life and utilization as a health food or animal feed. Microbiological limits of safety for a product are total aerobic plate counts (<10,000 CFU/gm), coliform bacteria (<100 CFU/gm) *E. coli* bacteria (<10 CFU/gm) counts, and *Salmonella* (negative).

The stabilization methods considered earlier were refrigeration, treatment with various chemicals, irradiation, and heating. All these technological processes had technical and economic limitations. Most current stabilization processes used in the U.S. use moisture added or dry extrusion technology (Hargrove, 1994), but none of them worked out to be beneficial for utilizing the bran. Our research efforts developed a unique non-chemical technology (The Rice<sup>TM</sup> Co., Proprietary Technology) by which the lipases and the lipoxygenases in the rice bran are exclusively inactivated. It meets the previously listed criteria of stabilization, preservation of the bioactives, improved shelf life (at least for one year), and possible utilization as a health food.

The hydrolytic stability and retention of endogenous antioxidants of the proprietary stabilized rice bran were studied at ambient temperature by Shin et al. (1997). It was shown that extrusion of rice bran below 120°C and no post-extrusion holding at elevated temperatures offers a protection against hydrolytic rancidity and a greater protection of endogenous antioxidants for a period of one year. Accelerated stability studies were carried out on 10 different lots of stabilized rice bran samples, incubated in an environmental chamber and maintained at 45°C and 80% RH for 90 days. Accelerated stability studies for three months are equivalent to a year at ambient temperature. Samples were drawn on 0, 45, and 90 days and were analyzed for bacterial load, free fatty acids, tocots, and  $\gamma$ -oryzanol (Reddysastray and Rukmini, 1997). The results indicated that free fatty acids increased initially within 45 days to 4%, and then a marginal increase to 5% in 90 days was observed. A progressive decrease of 6.7% in tocots in 45 days and a further loss of 17.8% in 90 days were observed. A marginal decrease of 11.8% in  $\gamma$ -oryzanol was observed in 90 days.

Hexanal is a measure of oxidative stability of low-fat foods like rice bran (Fritsch et al., 1977). Three different lots from a proprietary method stabilized rice bran in duplicate samples along with two different sources of stabilized rice bran were incubated at 45°C for 10 weeks. The samples were tested for hexanal by Headspace GC at 0, 2, 4, 6, 8, and 10 weeks. Stabilized rice bran by proprietary technology showed minimal hexanal (<1.0 ppm) at the end of 10 weeks. The other two samples of rice bran stabilized by different methods showed high hexanal values (<400 ppm) in 10 weeks. In another study, a by-product of stabilized rice bran (RiceX<sup>TM</sup> Solubles) was incubated at 37.8°C, and 25% RH for 20 weeks yielded <2.0 ppm hexanal. An increase in hexanal

to 5.0 ppm indicates a significant and unacceptable deterioration and rancidity (Fritsch, 1977).

Stabilization of rice bran by the proprietary technology improved the quality of the bran with longer shelf-life, nutrient availability, and microbiological safety. Several tailor-made nutraceutical products were prepared from stabilized rice bran. Stabilized rice bran is fractionated by a non-chemical process, using an enzyme and water, to create a water-soluble fraction (RiceX™ Solubles) and a water insoluble fraction.

## **FIBER FRACTIONS**

Fiber complexes are separated by centrifugation and dried to solid powders. The water-soluble fraction is a unique blend of water-soluble vitamins in high concentrations, including inositol, B vitamins, and water-soluble non-starchy polysaccharides. It contains soluble fiber and other micro-nutrients that are present in rice bran. It is suitable for utilization as drink-based formulations in diets and as food supplements in specific health conditions. The fiber concentrate is rich in insoluble fiber (42%) in addition to fat, fat-soluble vitamins, and protein. This product is useful in the functional food industry, especially for cholesterol reduction. The macro- and micro-nutrient compositions of all these tailor-made nutraceuticals are given in Table 1. As can be seen from the table, all these products are rich in nutrients. Some of the micro-nutrients, such as niacin, riboflavin, tocopherols, tocotrienols, and minerals like magnesium, potassium, and phosphorous are available at levels higher than the Recommended Daily Intake (RDI). Significant quantities of antioxidants, such as  $\gamma$ -oryzanol and phytosterols, are present in the derived rice products (Tables 2, 3, 4). The carbohydrates and fiber are of good quality and are comparable to oat bran.

The carotenoid content of stabilized rice bran is not very significant. The total carotenoids are 130 mcg/100 g. The carotenoid profile is good, as there are significant amounts of lycopene, lutein, and zeaxanthine, apart from  $\beta$ -carotene, which are all potent antioxidants.

## **PROTEIN**

Stabilized rice bran products are good sources of protein. The protein is complete. The most limiting amino acids are threonine and isoleucine (Hettiarachchy, 1994; Prakash, 1996). The protein efficiency ratio (PER) is 2.0, and the protein is hypoallergenic (Helm et al., 1996).

There are nearly 75 antioxidants identified in stabilized rice bran (Table 5). Most of them were studied earlier by Juliano (1985). The biological activity

TABLE 1. Nutrient composition of rice products.

Nutrients	Stabilized Rice Bran	Water-Soluble Derivative of Stabilized Rice Bran	Water-Insoluble Derivative of Stabilized Rice Bran
<b>Macronutrients (%)</b>			
Protein	14.5	7.5	20.5
Fat	20.5	26.5	13.5
Total Dietary Fiber	29.0 (Soluble fiber 2-6%)	3.0 (Soluble Fiber)	42.0 (Soluble fiber 0-1%)
Carbohydrates (available)	22.0	54.5	0.5
Ash	8.0	5.0	10.0
Moisture	6.0	3.0	3.5
<b>Micronutrients (mg/100g)</b>			
<b>Water-Soluble Vitamins</b>			
B-Vitamins			
Thiamin	2.65	3.64	2.0
Riboflavin	0.28	0.46	0.19
Niacin	46.87	76.6	30.55
Pantothenic acid	3.98	5.82	1.90
Vitamin B6	3.17	5.81	1.67
Biotin	0.014	0.015	0.011
Inositol	149.6	149.0	192.3
Fat-Soluble Vitamins			
Vitamin E			
Tocopherols and Tocotrienols (mg/100g)	25.61	18.0	3.73
Total Carotenoids (mg/100g)	0.13	ND <sup>a</sup>	ND <sup>a</sup>
γ-Oryzanol (mg/100g)	245.15	248.1	174.1
Total phytosterols (mg/100)	302.0	385.0	317.2
Minerals (mg/100g)			
Sodium	8.0	15.75	16.0
Calcium	39.7	8.33	92.5
Potassium	1573.0	1562.0	1670.0
Phosphorous	1591.0	763.0	2330.0
Zinc	5.5	1.8	9.4
Magnesium	727	170.9	1223.3

<sup>a</sup> ND—not detected.

and the antioxidant activity of these antioxidants are reviewed in the subsequent pages.

Rice bran oil is obtained from stabilized rice bran and is considered to be a high-quality health oil, because of its rich phytonutrient content. Most of the biological work carried out to date has been on rice bran oil since it

TABLE 2. Vitamin B content of rice product fractions.

B Vitamins	RDI (Recommended Daily Intake)	Solubilized Rice Bran Oil	Rice Bran Oil Solids	Fiber Fraction
Niacin	20.0 mg	47.0 mg/100 g	77.0 mg/100 g	31.0 mg/100 g
Pyridoxin	2.0 mg	3.2 mg/100 g	5.8 mg/100 g	1.7 mg/100 g
Biotin	400 µg	14.0 µg/100 g	15.0 µg/100 g	11.0 µg/100 g
Thiamin	1.5 mg	2.7 mg/100 g	3.6 mg/100 g	2.0 mg/100 g
Inositol		150 mg/100 g	149 mg/100 g	131 mg/100 g

Note: based on RiceX product fractions.

TABLE 3. Vitamin E and  $\gamma$ -oryzanol content of rice product fractions.

Rice Bran Products	Tocopherols (T)	Tocotrienols (T3)	Tocols (T + T3)	$\gamma$ -Oryzanol
Stabilized rice bran	12.0 mg/100 g	13.6 mg/100 g	25.6 mg/100 g	220-270 mg/100 g
Water-soluble derivative	8.0 mg/100 g	10.0 mg/100 g	18.0 mg/100 g	200-300 mg/100 g
Water-insoluble derivative	1.2 mg/100 g	2.5 mg/100 g	3.7 mg/100 g	200-300 mg/100 g
Rice bran oil	53 mg/100 g	54 mg/100 g	110 mg/100 g	1.4%
Max'E'™ rice bran oil	161 mg/100 g	189 mg/100 g	350 mg/100 g	2.2%

Note: based on RiceX product fractions.

TABLE 4. Phytosterols content of rice product fractions.

Phytosterols (mg/100 g)	Stabilized Rice Bran	Rice Bran Soluble Fraction	Fiber Fraction
$\beta$ -Sitosterol	152	212	147
Campesterol	92	117	90
Stigmasterol	60	70	67
Brassicasterol	15	15	13
Total phytosterols	302	385	317

Note: based on RiceX product fractions.

TABLE 5. Antioxidants in riceX™ stabilized rice bran.

<b>γ-Oryzanol</b> 2200–3000 ppm	<b>Tocopherols/Tocotrienols</b> 220–320 ppm	<b>Polyphenols</b>	<b>Minerals</b>
Cycloartenyl ferulate	α-Tocopherol	Ferulic acid	Magnesium (6250–6440)
24-methylene	β-Tocopherol	α-Lipoic acid	Calcium (303–500)
Cycloartenyl ferulate	γ-Tocopherol	Methyl ferulate	Phosphorous (14700–17000)
Campesteryl ferulate	δ-Tocopherol	p-Coumaric acid	
β-Sitosteryl ferulate	λ-Tocotrienol	p-Sinapic acid	
Stigmasteryl ferulate	β-Tocotrienol	Isovitexin	
	γ-Tocotrienol	Proanthocyanidins	
δ-Tocotrienol			
<b>Polysaccharides</b>	<b>Carotenoids 0.9–1.6 ppm</b>	<b>Amino Acids</b>	
Cycloartenol ferulic acid	λ-Carotene	Tryptophan (2100)	
Glycoside	β-Carotene	Histidine (3800)	
Diferulic-acid complex	Lycopene	Methionine (2500)	
Diferulic-acid-3 Glucose-2-calcium complex	Lutein	Cysteine (336–448)	
	Zeaxanthine	Cystine (336–448)	
		Arginine (108000)	
<b>Phytosterols 2230–4400 ppm</b>	<b>Phospholipids</b>	<b>Enzymes</b>	<b>B-Vitamins</b>
β-Sitosterol	Phosphatidylcholine	Glutathione peroxidase	Thiamin (22–31)
Campesterol	Phosphatidylcholine	Methionine reductase	Riboflavin (2.2–3.5)
Stigmasterol	Ethanolamine	Superoxidase dismutase	Niacin (370–660)
5 Avinsterol	Lysolecithin	Polyphenol oxidase	Pantothenic acid (36–50)
7 Stigmasterol		Aspartate amino transferase	Pyridoxin (29–42)
Isofucosterol		Isozymes AAT-1, AAT-2	Inositol/myoninositol (1200–1880)
Gramisterol		Coenzyme Q10	Biotin (0.1–0.22)
Citrostadienol			Choline (930–1150)
Obtusifolol			Phytates (1500–1710)
Branosterol			
28-Homotyphasterol			
28-Homosteasteronic acid			
6-Deoxycastasterone			
β-Amyrin			

contains all the concentrated bioactives of rice bran except the B-complex vitamins. The unsaponifiable fraction of the oil contains many more micro-nutrients, such as phytosterols, polyphenols, squalene, and several unidentified compounds, which are under investigation. The fatty acid profile of rice bran oil and peanut oil are given in (Table 6). There are several value-added nutraceutical derivatives, which are obtained during rice bran oil processing. They have considerable commercial value due to their biological importance. The by-products obtained during the processing of rice bran oil are listed below.

- High-quality wax: is equivalent to carnauba wax (3–5% of crude oil) for cosmetic products.
- Lecithins: phospholipids (1.0–2.0% of crude oil) are used as antioxidants in food and feed industry and other industrial uses.
- Soap stock: considerable amount of low-grade oil is trapped with the sodium salt of the fatty acids of rice bran. Up to 4% is trapped in the soap stock, which is a valuable raw material for the soap industry.
- $\gamma$ -Oryzanol: a potent antioxidant and a valuable nutraceutical product, 1.0 to 1.5% can be recovered from the soap stock.
- Defatted rice bran: rich in B-vitamins, minerals, and fiber, used as a valuable functional food.

Several bioactive compounds have been identified in the above-designated rice bran products. The major bioactive groups having proven health benefits that have been identified and quantitated include: carotenoids, B vitamins, inositol, vitamin E—tocopherols and tocotrienols,  $\gamma$ -oryzanol, phytosterols, polyphenols, minerals, lecithins, fiber and non-starchy polysaccharides, protein and amino acids, and fat and fatty acids.

The biological role of the major antioxidants, such as vitamin E and its

TABLE 6. Fatty acid profile of rice bran oil and peanut oil.

Fatty Acid Profile (%)	Rice Bran Oil Units	Peanut Oil Units
Palmitic 16:0	16.0	15.0
Stearic 18:0	2.0	3.1
Oleic 18:1	42.0	42.6
Linoleic 18:2	36.6	35.9
Linolenic 18:3	1.5	nil
Arachidic 20:0	nil	2.2
Behenic 22:0	nil	1.0
Total saturated fatty acids	18.0	21.3
Monounsaturates	42.0	42.6
Polyunsaturates	38.1	35.9
$\gamma$ -oryzanol (ppm)	14,000–15,000	nil
Total T + T3 (ppm)	1200–1500	nil

isomers,  $\gamma$ -oryzanol, phytosterols, and polyphenols, are discussed in detail below. Natural foods like rice bran, containing several antioxidants, elicit antioxidant activity as a synergistic function of all the bioactive molecules rather than the effect from single molecules. The bioactives have several modes of action at the molecular level:

- antioxidant activity
- inhibition of Phase 1 microsomal enzymes
- activation of Phase 2 microsomal enzymes
- competition with active binding sites—studies in animal models
- competitive inhibitors—animal models and cell lines
- cell regulation and trans-cellular signaling

## ANTIOXIDANT EFFECT

Antioxidant defense mechanisms in a biological system play a major role in the prevention of a number of diseases, including cardiovascular, cerebrovascular, many age-related disorders, and some forms of cancer (Garewell, 1997). Reactive oxygen species can produce highly reactive secondary oxidation products causing diseases such as atherosclerosis, ischemia of the brain and heart, cancer, diabetes, infection, aging, radiation damage, frost bite, arthritis, inflammation, shock, and Parkinsonism. Endogenous biological oxidants formed during metabolism and antioxidants entering from exogenous environmental sources, such as cigarette smoke, ozone and ultraviolet light, are potentially dangerous because they alter normal functions and they can damage cellular components. There is a delicate balance between oxidant and antioxidant loads in the biological systems. Maintaining good health and preventing diseases is a constant battle by the defense and immune systems of the body. Epidemiological evidences are mounting on the significant role of natural antioxidants, which play a vital role in maintaining and preventing disease (Packer, 1995). The antioxidant functions of rice bran products include:

- radical scavengers—tocopherols, carotenoids,  $\gamma$ -oryzanol
- hydrogen donors—polyphenols, ferulic acid tocopherols
- electron donors— $\gamma$ -oryzanol, tocopherols, polyphenols
- peroxide decomposers—peroxidases as glutathione peroxidase
- singlet oxygen quenchers—tocopherols and carotenoids
- enzyme inhibitors—tocotrienols, ferulic acid, and  $\gamma$ -oryzanol
- metal chelators—protein and aminoacids, phytates

## INHIBITION OF PHASE 1 ENZYMES

There is a group of microsomal enzymes known as Phase 1 enzymes that

are carcinogenic metabolizing/activating enzymes. These enzymes activate the biological molecules into reactive metabolites, which directly attack the vital components of the cell and activate carcinogenesis. Polyphenols in rice bran products are known to inhibit Phase 1 enzymes (Wood et al., 1982). Antimutagenic and anticarcinogenic activity of rice bran oil was evaluated in Ame's bacterial assay system (Rukmini and Kalpagam, 1985).

## ENHANCEMENT OF PHASE 2 MICROSOMAL ENZYMES

Another group of microsomal enzymes is known as Phase 2 enzymes, belong to the detoxifying system. These enzymes have the capability of detoxifying the potential carcinogen into a glucuronide, which is eventually excreted from the system. The bioactives molecules, such as ferulic acid, are known to enhance these enzymes several-fold leading to the effective elimination of carcinogenesis (Graf, 1992). Antioxidants can function at any stage of carcinogenesis, either at the initiation stage or promotion stage or even at the tumor production stage, by the repair mechanism, arresting the replication of tumor cell. The bioactives in rice bran products act as preventive antioxidants, suppressing the free radical formation as radical scavenging antioxidants and repair antioxidants. As mentioned, above the tocopherols, tocotrienols, polyphenols, and  $\gamma$ -oryzanol were studied in detail.

## COMPETITION WITH ACTIVE BINDING SITES

The dietary phytochemicals/bioactive molecules present in rice bran sometimes have similar structures to the biochemical components of the system. As an example, cycloartenol, a component of  $\gamma$ -oryzanol, has a similar structural configuration as cholesterol (Figure 1), a biochemical metabolite in the body. Thus, cycloartenol competes with the active binding sites of cholesterol in the liver and sequesters cholesterol from the system, resulting in a hypocholesterolemic effect (Zambotti et al., 1975). Many structural similarities of the bioactive molecules facilitate the sequestration of harmful products from the body. This results in cycloartenol competing with the binding sites of cholesterol in the liver and sequestering cholesterol, which is excreted as bile acids and bile pigments in the urine. Rats fed rice bran oil, which is rich in  $\gamma$ -oryzanol, excreted higher levels of bile acids and bile pigments than the rats fed peanut oil as the control (Figure 2) (Sharma and Rukmini, 1986).

## COMPETITIVE INHIBITORS

Some of the bioactive molecules are competitive inhibitors of the enzyme

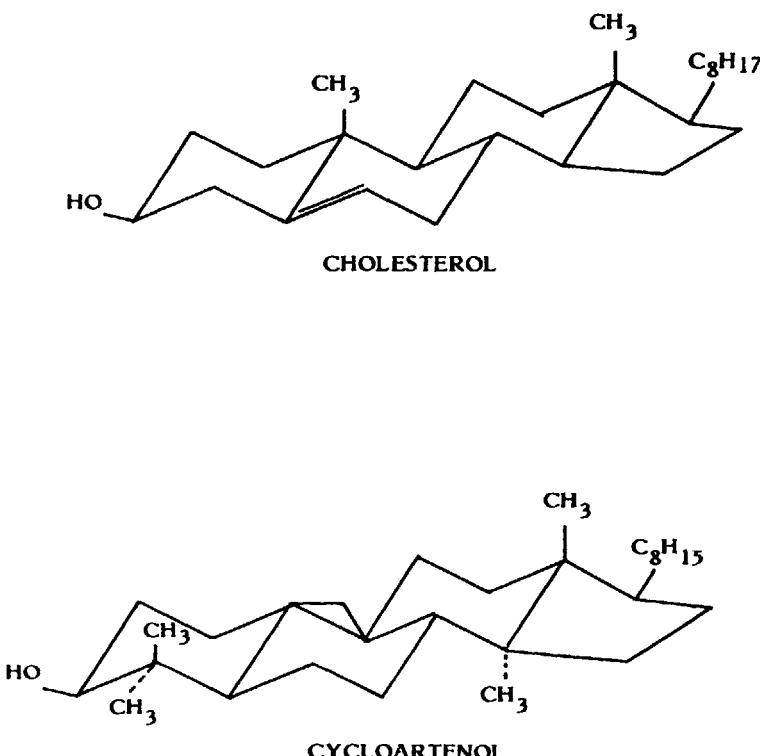
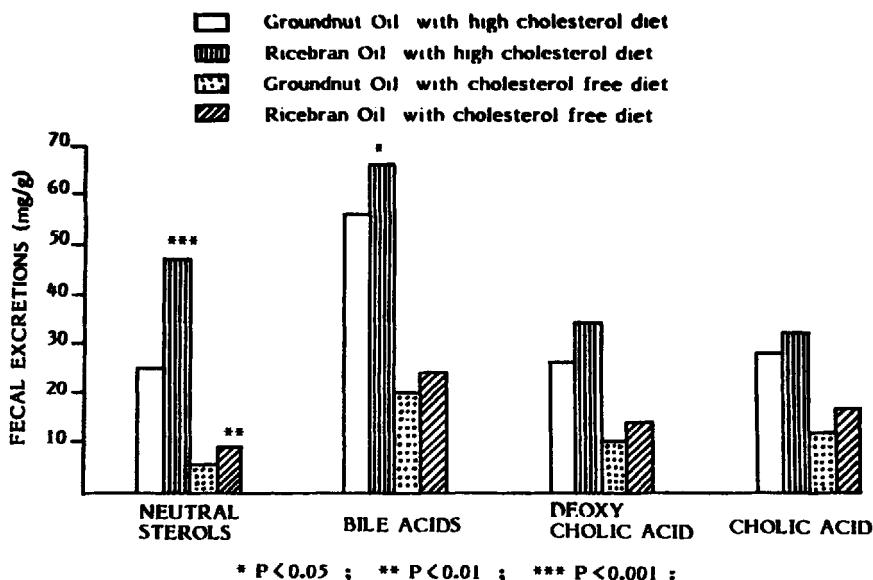


Figure 1 Structural similarity of cholesterol and cycloartenol.

systems, especially the carcinogenic activating enzymes. Thus, the harmful enzymes are not activated, and the carcinogens are sequestered from the system. HMGCoA reductase is an enzyme involved in the biosynthesis of cholesterol and is inhibited by tocotrienols of rice bran. This results in the reduced synthesis of cholesterol (Qureshi, 1986). The inhibition of the enzyme HMGCoA reductase was demonstrated in humans by the feeding of rice bran and rice bran oil (Hegsted and Kousik, 1994).

## CELL REGULATION AND TRANSCELLULAR SIGNALING

The condition that occurs when eukaryotic cells are exposed to above-normal levels of reactive oxygen species (ROS) is referred to as oxidative stress. This phenomenon occurs frequently in cells exposed to UV light (Devary et al., 1992), ionizing radiation (Datta et al., 1992), and certain endogenous ROS conditions resulting in tumor promotion (Zimmerman and Cerutti, 1984).



**Figure 2** Fecal excretion of neutral sterols and bile acids in rats fed rice bran oil and groundnut oil.

Antioxidants help in cell regulation and cellular signaling, resulting in the prevention of carcinogenesis (Lin et al., 1995). Bioactives of rice bran products, inositol hexa-phosphate, are shown to effect cell regulation and cellular signaling in the prevention of carcinogenesis (Shamsuddin, 1995).

## PHYSIOLOGICAL SIGNIFICANCE OF MAJOR BIOACTIVES IN RICE BRAN

### CAROTENOIDS

Carotenoids in stabilized rice bran are 130 mcg/100 g, which is not a significant dietary amount. But as apart of the antioxidant system in rice bran products, especially with tocopherols, its physiological role increases by several fold as suggested in the literature (Krinsky, 1993).

### VITAMIN B

Niacin, thiamin, pyridoxin, biotin, and inositol are the B vitamins present in significant amounts in stabilized rice bran and its products (Table 2). The physiological role of these vitamins in carbohydrate and amino acid metabo-

lism is well documented in literature (Rindi, 1996). Rice bran products are rich in niacin (Table 2). Niacin is important for maintaining blood sugar, intracellular energy production, and controlling hyperglycemia. Pyridoxin is a vital component for the prevention of peripheral neuropathy in diabetics. Biotin acts in the initial step of glucose utilization by the cell. Biotin may also play a role in stabilizing blood glucose. Biotin appears to have a role in the management of diabetes (Mock, 1996). The health effects of these products as nutritional support to diabetic patients to correct glucose metabolism and prevent peripheral neuropathy is being explored. Inositol is a natural antioxidant. It was shown to reduce cholesterol, inhibit platelet aggregation, reduce fatty liver, and enhances insulin secretory process. Shamsuddin et al. (1997) recently reviewed the suppression of liver, skin, colon, and breast tumors by inositol. Thus, the vitamin Bs seem to help diabetic health and inhibit different forms of cancers. Rice bran and its products are rich in B vitamins, more than the RDI, and may help in the prevention of disease conditions.

## VITAMIN E

Vitamin E is a collective name given to a group of naturally occurring tocopherols and tocotrienols found abundantly in plants and plant oils. Both tocopherols and tocotrienols have identical structures with a chromanol head group and phytol- and farnesyl-side chains respectively (Figure 3).

The hydrophobic tail, the anchor for vitamin E molecules into membranes or in lipoproteins, differs so that the tocopherols have a complete saturated side chain while the tocotrienols have three unsaturated linkages in the tail. Vitamin E and its isomers ( $\alpha, \beta, \gamma, \delta$ -tocopherols and  $\alpha, \beta, \gamma, \delta$ -tocotrienols) are potent lipophilic antioxidants, similar to carotenoids. Additionally, they all possess varying degrees of vitamin E activity.

Tocopherols are powerful antioxidants and have potent vitamin E activity. Tocopherols have higher protective activity against cardiotoxicity (Ozer et al., 1993). The bioabsorption of various stereoisomers of vitamin E indicates a large degree of discrimination and selectivity. In human supplementation studies on the absorption of tocotrienols and tocopherols from the intestinal tract into the chylomicrons fractions, the subsequent appearance in human lipoproteins indicates the presence of specific vitamin E tocopherol-binding protein, while regulating vitamin E metabolism in the hepatocytes (Meydani, 1995).

Tocotrienols (T3) have been shown to exert a stronger anti-tumor action (Nesaratnam et al., 1998). Tocotrienols inhibit the liver microsomal enzyme HMGCoA reductase, a key enzyme involved in the cholesterol biosynthetic pathway. This results in the reduction of circulating cholesterol, LDL-C, apo-B, thromboxane B2, and platelet factor 4 in animal and human systems (Qureshi and Qureshi, 1992).  $\gamma$ -T3 and  $\delta$ -T3 are very effective for hypcholesterolemic action and thromboembolic disorders.

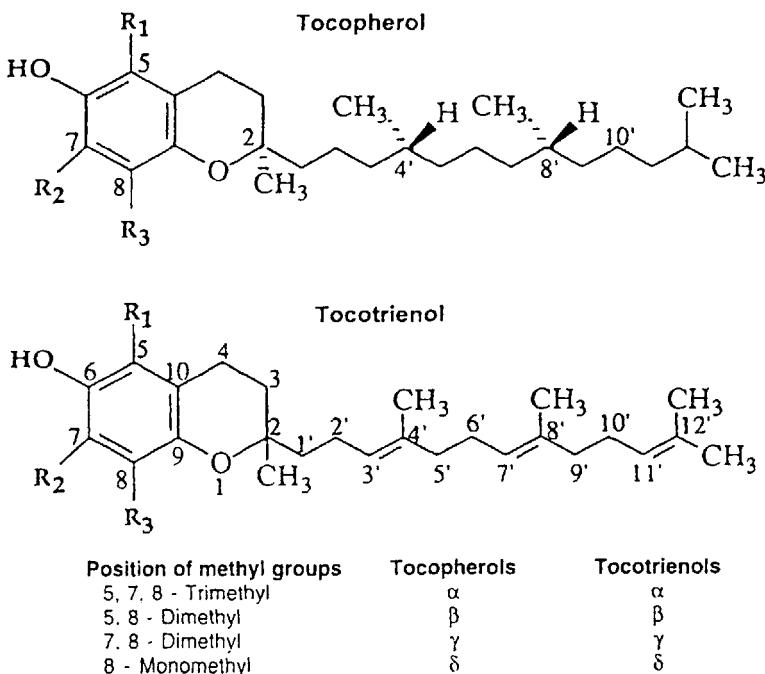


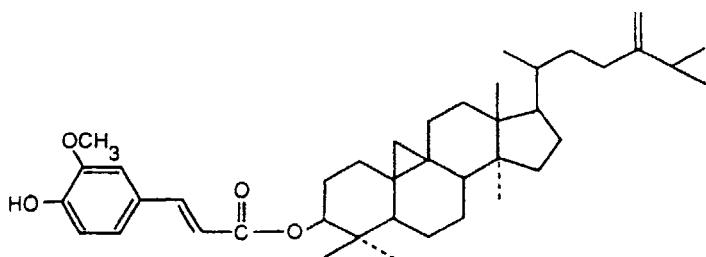
Figure 3 Structure of tocopherols and tocotrienols.

### $\gamma$ -ORYZANOL

$\gamma$ -Oryzanol is a unique antioxidant present in rice bran and its products. Chemically, it is a mixture of ferulic acid esters of triterpene alcohols and phytosterols (Figure 4).

- cycloartenyl ferulate
- 24-methylene cycloartanyl ferulate
- campesteryl ferulate
- stigmasteryl ferulate
- $\beta$ -sitosteryl ferulate

Ferulic acid by itself is a potent antioxidant (Graf, 1992).  $\gamma$ -Oryzanol is an equally powerful antioxidant. It has the ability to absorb UV light and is used in skin protection creams (Morita, 1986). It is a potent hypolipidemic agent (Yoshino et al., 1989; Lichtenstein et al., 1994) and antiatherogenic agent, because it inhibits platelet aggregation, inhibits aortic streaks (Seetharamiah and Chandrasekhara, 1990; Rong et al., 1997), inhibits LDL oxidation, and is a potent lipotropic agent (Oliver, 1984). Lipid peroxidation has been shown to be prevented in the retina by  $\gamma$ -oryzanol, because of its antioxidant property



**Figure 4** Chemical structure for  $\gamma$ -oryzanol (24-methylene-cycloartanol ester of ferulic acid).

(Fukushi, 1996). It is a potent neuroregulator, acting on the autonomic nervous system (Nakazawa et al., 1977), and it acts as an anabolic steroid by improving the lean body mass (Bruni, 1988). It is also known to be antimutagenic and anticarcinogenic (Rukmini and Kalpagam, 1985; Tamagava et al., 1992; Tsushima et al., 1991).

## PHYTOSTEROLS

Phytosterols are present in significant amounts in stabilized rice bran and its products (Table 4). Phytosterols, having similar structure to cholesterol, compete with the uptake of dietary cholesterol in the intestines and facilitate its excretion from the body. Cholesterol is, thus, metabolized into bile acids and bile salts. The phytosterols trap the bile acids and bile salts and prevent them from reconverting to cholesterol. Thus, phytosterols alone or combined with dietary measures offer a non-medical approach to the reduction of plasma cholesterol for those in whom its elevation warrants treatment by drug or diet (Weststrate, and Meijir, 1998). Phytosterols also appear to block the development of tumors in colon, breast, and prostate glands (Berges et al., 1995). Phytosterols appear to alter cell membrane transfer in tumor growth and reduce inflammation.

## POLYPHENOLS

In addition to vitamin E, the majority of polyphenols are cinnamic acid derivatives. Ferulic acid is abundantly found in rice bran, both in the free form and as an ester of arabinoxylans, diferulic acid esters, and esters with phytosterols and triterpene alcohols.  $\rho$ -Coumaric acid,  $\alpha$ -lipoic acid, and sinapic acid were also detected in rice bran. Stabilized rice bran has 0.1% lipoic acid. Total polyphenols were quantitated in rice bran and were reported as

85.6 mg/kg, with trans-ferulic acid the most abundant compound, averaging 75.1 mg/kg (Ramaratnam et al., 1986). Phenolic antioxidants act to inhibit lipid oxidation by trapping peroxy radical (Chim et al., 1991). Polyphenols have the ability to block specific enzymes that cause inflammation. They also modify the prostaglandin pathway and thereby protect platelet aggregation. In 1995, Fiala et al. indicated the beneficial effects of naturally occurring polyphenols in the food we eat.

## FIBER

Rice bran and its products are major contributors of dietary fiber. Implications of dietary fiber are well defined (Kritchevsky and Bonfield, 1995). The most widespread, extensively advertised and consumed fiber is from cereals. Dietary fiber sometimes traps natural antioxidants and acts as an antioxidant dietary fiber (Saura-Calixto, 1998). The major bioactives in rice bran are discussed above briefly, and the detailed functionality of these bioactives are discussed in detail under the health effects.

## **HEALTH EFFECTS OF STABILIZED RICE BRAN AND ITS PRODUCTS**

There are several health effects in which rice bran has been implicated in the literature. However, clear studies are available for the first three health conditions, which are discussed in detail.

- cardiovascular disease/atherosclerosis/cholesterol metabolism
- diabetes/glucose metabolism
- cancer
- liver abnormalities
- hypertension/control of high blood pressure
- arthritis
- skin nutrition
- neurological abnormalities
- obesity

Scientific evidence in the literature indicates that stabilized rice bran and its products are known to have specific beneficial effects in the prevention of cardiovascular diseases and improve postprandial glucose responses, besides correcting several health disorders listed above. These health effects are the synergistic function of the several bioactives present in stabilized rice bran products. Results of several animal and clinical studies are available in the literature and are discussed below to support the synergistic effects of the various bioactives present in the stabilized rice bran products in significant

amounts. These findings may lead to some understanding of the mechanism involved, leading to the several health effects discussed.

## CARDIOVASCULAR DISEASE

Cardiovascular disease includes arteriosclerosis, atherosclerosis, and xanthomatosis in humans. The primary cause and the major risk factor for this disease is hypercholesterolemia. Hypercholesterolemia is a condition where the circulating total cholesterol, LDL-cholesterol, and triglycerides are high (NCEP/National Cholesterol Education Program as the yardstick). This condition is also known as hyperlipidemia. Hyperlipidemia or hypercholesterolemia predisposes individuals to cardiovascular disease. Lipoprotein particles have a surface protein that governs their receptor-mediated uptake. Cholesterol and LDL-C tend to get oxidized. The oxidized cholesterol and LDL-C do not recognize their receptors and, hence, form foam cells, which are deposited on the smooth muscle of the intima of the arterial walls. This results in the narrowing down of the arteries and restricting the flow of blood to the heart. The narrowing is due to the formation of plaques or streaks or clumps of platelets, derived from cholesterol, oxidized LDL, foam cell of VLDL, fibrous tissues, and decaying cells in the inner lining of the arteries. This condition is conducive for the formation of thrombi (blood clots), which can break off and form emboli. The emboli travel through the blood stream and can block the arterial vessels. Because the blood supply is restricted to the heart, this condition will lead to atherosclerosis, or arteriosclerosis and heart attacks. The major preventive care is to keep cholesterol and the other lipid parameters under control and at normal levels to prevent cardiovascular diseases.

There are several animal experiments and clinical studies in the literature using rice bran oil or rice bran, which resulted in significant hypocholesterolemic effect. Sharma and Rukmini made the first observation of the hypocholesterolemic effect of rice bran oil in 1986. Rats fed a diet containing 10% rice bran oil for 28 days demonstrated a significant reduction in total cholesterol and triglycerides (Figure 5). The same authors subsequently reported in 1987 that the unsaponifiable portion of rice bran oil alone, in the amount present in rice bran oil, demonstrated significant hypocholesterolemic effect in a rodent model. The authors concluded that the micro-nutrients present in the unsaponifiable portion of rice bran oil are responsible for the hypocholesterolemic effect. Subsequently, several researchers supported the earlier observation of Sharma and Rukmini (Sugano and Tsuji, 1997; Lichtenstein et al., 1994; Nicolosi et al., 1991; Hegsted and Kousik, 1994; Orthoefer, 1996). Rice bran oil is a concentrated source of most of the bioactives present in rice bran. Hence, most of the earlier research was done on rice bran oil. Several papers were published on the cholesterol-lowering effect and other nutritional effects in Japan and India (Rukmini and Raghuram, 1991; Purushothama et al., 1995;

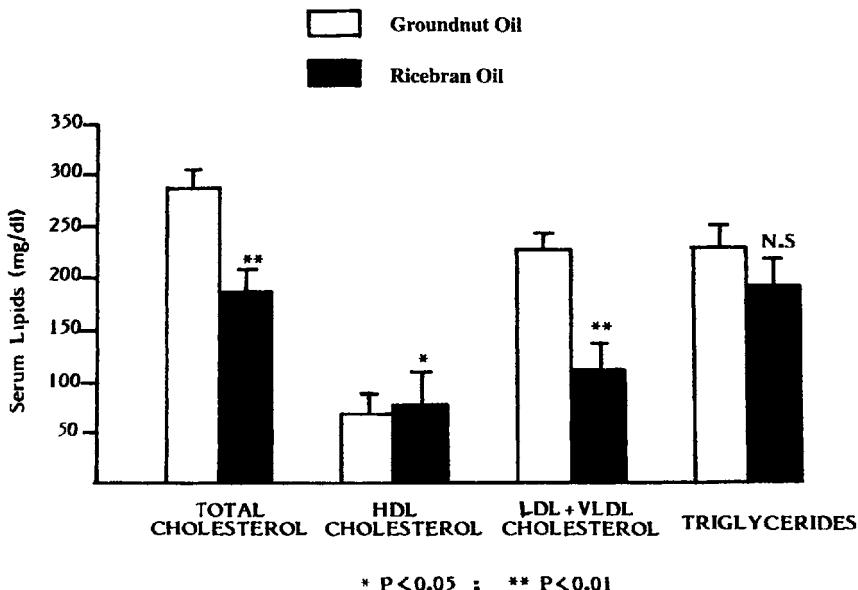
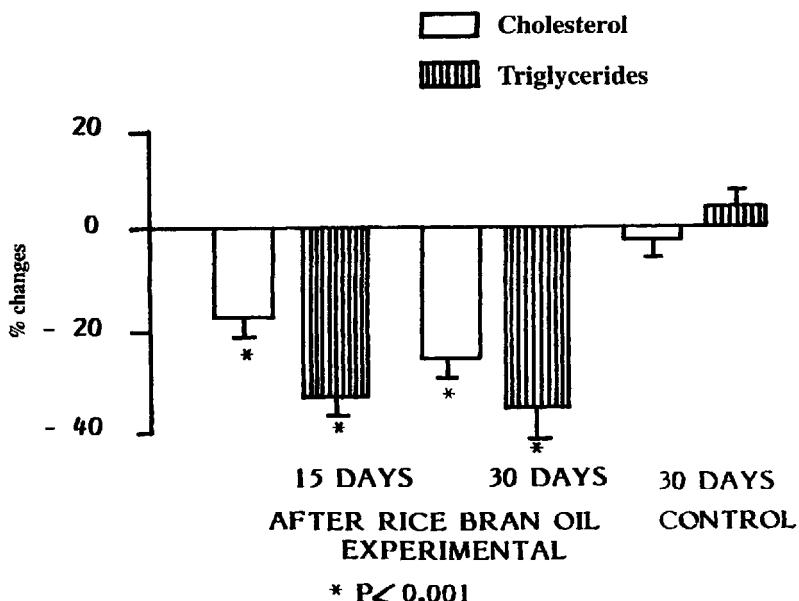


Figure 5 Lipid profile of rats fed rice bran oil and groundnut oil.

Beg et al., 1996; Seetharamiah and Chandrasekhara, 1989). Suzuki et al. (1984) conducted a study on 80 normocholesterolemic subjects for 10 weeks, where several oils with varying degrees of polyunsaturated fatty acids such as peanut, cottonseed, soybean, sunflower, safflower, palm, rapeseed, coconut, and rice bran oils were incorporated in the diet (80 g/day/person). Initial and final serum cholesterol and lipid levels were monitored in these subjects. The authors compared the lowering of cholesterol levels of several oils, including rice bran oil. The results of the study indicated that rice bran oil is the most potent of all the oils, showing 17 times greater cholesterol lowering effect than cottonseed oil. These authors subsequently showed that the micro-nutrients in the unsaponifiable fraction are responsible for the cholesterol reduction, more than the polyunsaturated fat. They have also shown that a combination of the micro-nutrients of the rice bran oil and the high polyunsaturated fat of safflower oil in a ratio of seven to three can bring down cholesterol levels to normal level in hypocholesterolemic subjects within 7 days (Suzuki and Oshima, 1970). Raghuram et al. (1989) carried out a clinical study with rice bran oil in hypocholesterolemic subjects and showed that dietary rice bran oil has a significant hypocholesterolemic effect. In their study, 12 hypercholesterolemic subjects were maintained with dietary rice bran oil (30–35 g/day/person) for 30 days. Significant reductions in total cholesterol (−17.5% in 15 days; −26%



**Figure 6** Changes in serum cholesterol and triglyceride levels in human subjects fed rice bran oil.

in 30 days) and triglycerides ( $-32.4\%$  in 15 days;  $-39\%$  in 30 days) were observed in 30 days (Figure 6). Gerhardt and Gallo in 1998 have showed that full-fat rice bran reduces serum cholesterol and LDL-C as much as oat bran in humans. Hegsted and Windhauser (1993) and Hegsted and Kousik (1994) demonstrated the hypcholesterolemic effect of rice bran as well as rice bran oil in humans. Rong et al. (1997) and Seetharamiah and Chandrasekhara (1988) fed  $\gamma$ -oryzanol to rats and demonstrated inhibition of aortic streaks, inhibition of platelet aggregation, and modulation of prostaglandins, accompanied by significant hypolipidemic effect. All the above-cited animal and human experiments indicate that rice bran and rice bran oil are hypcholesterolemic in animal models and humans. As we look into the macro-nutrient and micro-nutrient content of rice bran and rice bran oil, the synergistic effect of all the nutrients may be responsible for the biological effect. The major components of the unsaponifiable fraction are significant amounts of polyphenols, phytosterols, tocopherols, and tocotrienols (80–110 mg %),  $\gamma$ -oryzanol (1–1.3 g %), in addition to minor antioxidants. Some of the mechanisms operating in the hypolipidemic effect as understood from several animal experiments are discussed below.

- (1) Enzyme inhibitions: Liver microsomal enzymes play a key role in cholesterol homeostasis. This includes endogenous cholesterol synthesis, storage, and excretion. HMGCoA reductase, the key enzyme involved in the biosynthesis of cholesterol, is inhibited by tocotrienols, which are present in rice bran and rice bran oil. This results in the lowered synthesis of endogenous cholesterol and is also highly effective in lowering LDL. The second enzyme, ACAT (acyl-coenzyme A: acyl transferase), esterifies cholesterol for storage within the cell or lipoproteins.  $\gamma$ -Oryzanol, present in rice bran and rice bran oil, inhibits this enzyme. Inhibiting ACAT and preventing the esterification of cellular cholesterol will facilitate cholesterol clearance. ACAT inhibition may have a three-fold impact: cholesterol enrichment of HDL, thereby increased HDL-C levels, lowered VLDL synthesis, and impaired intestinal absorption of cholesterol with a resulting decline in circulating LDL. The third group of enzymes are cholesterol esterases: The enzyme inhibition by  $\gamma$ -oryzanol in rice bran and rice bran oil results in the hydrolysis of cholesterol esters into free cholesterol, resulting in more free cholesterol for metabolism and excretion.
- (2) Antioxidant effect: The antioxidants, especially the high tocopherol content of rice bran and rice bran oil, prevent LDL oxidation. There are around 75 antioxidants identified in rice bran. The synergistic antioxidant activity plays a role in controlling many of the adverse biological activities leading to atherosclerosis.
- (3) White cell reactions: Inhibition of platelet aggregation, aortic streaks, and inhibition of leucotrienes affect the macrophage function by reducing chemotaxis, which have a positive impact on atherogenesis. It has been shown by Seetharamiah and Chandrasekhara (1990) and Rong et al. (1997) that feeding  $\gamma$ -oryzanol to rats inhibits platelet aggregation, inhibits leucotrienes, and reduces aortic streaks.
- (4) Cholesterol mechanisms: Fiber and phytosterols present in rice bran and rice bran oil form a complex with cholesterol metabolites, creating an intestinal microflora, and the cholesterol is metabolized and excreted very fast so that it cannot get back into the circulation.
- (5) Aminoacids: Plant proteins with arginine/lysine ratios below 2.0 are known to help in maintaining the cholesterol homeostasis.

The above information definitely suggests that rice bran and rice bran oil have a great impact on reducing the cholesterol and lipid levels in the preventive care of atherosclerosis.

## **DIABETES AND GLUCOSE METABOLISM**

Diabetes is another metabolic disorder where the end product of ingested carbohydrate is not converted into energy, but the glucose is converted into

abnormal metabolites, leading to adverse effects and complications to vital organs. The causative factors are partly genetic and partly due to stress, modern lifestyle, and defective nutrition. B vitamins, especially pyridoxin and niacin, help in protecting diabetic complications. Diabetic patients are in need of strong antioxidant defenses to modulate the consequences of hyperglycemia.

Stabilized rice bran and its products, especially the water-soluble derivatives are loaded with high concentrations of B vitamins, water-soluble non-starchy polysaccharides, protein, high-quality fat with rich antioxidant concentration, inositol, and water-soluble minerals like potassium, magnesium, and potassium. All these components are known to play a key role at the molecular level in modulating the glucose kinetics in diabetics. A preliminary clinical evaluation to establish the antidiabetic effect of soluble rice bran fractions was carried out in 26 Type 1 and 31 Type 2 diabetic patients. The products were given to the subjects in two divided doses of 10 grams each, one taken before breakfast and one taken before dinner in milk/fruit juice for 8 weeks. The treatment demonstrated significant reduction ( $p < 0.5$ ) in serum fasting glucose in 8 weeks when compared to initial values, both in Type 1 and Type 2 diabetics (33%). The glycosylated hemoglobin also demonstrated a significant reduction ( $p < 0.5$ ) of 10% and 11% in Type 1 and Type 2 diabetics (Rukmini, 1998).

Rice bran products and rice bran oil have 75 antioxidants, hypoallergenic protein with a good amino acid make up, a good-quality fat, fiber, and non-starchy polysaccharides and are rich in B-complex vitamins, tocopherols, and tocotrienols in substantial quantities. The mineral magnesium is required as a cofactor to several enzymes and is shown to correct diabetes (Yajnik et al., 1984). Rice bran and its products may stabilize diabetes and may have a role in preventing diabetic complications.

## CANCER

Cancer is the fast growth of abnormal cells in various organs such as the liver, esophagus, colon, intestines, prostate, ovary, uterus, breast, and other vital organs. It is basically a function of unchecked free radical damage and partially an altered genetic manifestation. There are three stages of cancer: initiation, propagation, and tumor production. Cancer can be prevented at every stage by the antioxidants. In the initiation stage, the carcinogen gets activated into an active metabolite by group enzymes in the liver microsomes known as the Phase 1 carcinogenic-activating enzymes. Inhibition of Phase 1 enzymes results in the prevention of carcinogenesis. There is a latent period between initiation and promotion when the activated carcinogen finds a target tissue and interacts with cellular components forming abnormal complexes to proliferate. Liver microsomes have Phase 2 detoxifying enzymes, glutathione-S-transferase, which prevents this abnormal complex formation and converts

the carcinogen into a glucuronide and is eventually excreted. There is evidence to show that feeding rice bran oil to animals for three months caused an inhibition of Phase 1 enzymes and an elevation of Phase 2 enzymes (Manorama, 1993). In the third phase of carcinogenesis, the tumor formation stage, the free radicals play a crucial role. The carcinogen, by the attack of free radicals, undergoes chain reactions generating free radicals. The powerful antioxidant load in stabilized rice bran and rice bran oil, especially the polyphenols, tocopherols, tocotrienols, and  $\gamma$ -oryzanol with other minor antioxidants, are involved in the prevention of carcinogenesis.

## LIVER ABNORMALITIES

The liver is an organ endowed with a cadre of enzymes for food digestion and where foreign compounds in the body are detoxified. If the enzyme systems do not work properly, the liver cells become damaged, and continued regeneration of liver cells is important for its normal function. Inositol is known to help in the fast regeneration of liver cells. Inositol is a powerful antioxidant and is also known to inhibit cancer development.

There are other allied health effects carried out in the liver, such as regulation of blood pressure, inflammation, obesity, and neurologic pathways. These health effects of rice bran are reported extensively in the Japanese literature. Most of these effects are brought about by the antioxidants present in rice bran as well as the immuno-modulatory effect of rice bran. The mechanism of action is not clearly understood. However, further proof is necessary to establish the efficacy of rice bran in the above-mentioned disorders.

## SKIN NUTRITION

The  $\gamma$ -oryzanol present in rice bran or rice bran oil has a powerful UV absorbency and is used in skin lotions and sun tan creams. In addition to  $\gamma$ -oryzanol, vitamin E and squalene are present in rice bran and rice bran oil, which is responsible for skin nutrition. Rice bran and rice bran oil are used in cosmetic preparations for special skin care.

## CONCLUSION

Rice bran is rich in many potent bioactive molecules. These bioactive molecules have tremendous potential health benefits in humans and animals. For effective utilization of these biologically active components, stabilization of rice bran and assured stable shelflife is necessary. Stabilized rice bran by proprietary technology has a superior quality of nutrients and superior shelf life. Stabilized rice bran and its products are all good candidates for functional

foods. The tailor-made rice bran products appear to have a greater economic impact on the functional food and snack food industry. Several breakfast foods, cereals, baked products, and snack foods are developed from these products. Functional foods and designer foods are being formulated with the intent to use them as diet supplements for hypercholesterolemia, diabetes, prevention of cancer, obesity, and skin nutrition. Already, there are ample evidences in the literature for rice bran oil as a potent hypcholesterolemic agent. Studies indicate that rice bran products have potential benefits in the prevention of chronic diseases, such as atherosclerosis, diabetes, cancer, hypertension, and allied metabolic disorders. Studies are in progress to establish the sensory evaluation, acceptability of the formulations, and clinical efficacy of each rice bran product as a preventive functional food for various health conditions. However, the dosage of the products as a supportive therapy needs to be established. Commercial availability of the product and future potential also need to be established.

The above-mentioned biological effects are brought about by the synergetic effect of the several bioactive compounds present in rice bran and its products. Isolation of individual components may not be effective as they may lose their biological effects. Rice bran, an underutilized by-product, can be made into a highly nutritious, health promoting food for mankind.

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# Designing Functional Foods to Enhance Health

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## INTRODUCTION

A new diet-health paradigm has continued to evolve in recent years that places more emphasis on the positive aspects of diet. The paradigm goes beyond the well-defined role of food constituents as essential nutrients required for promoting growth and sustaining life to one that optimizes the quality of life, preventing or delaying the premature onset of chronic diseases (Bidlack, 1998). The amount and composition of food consumed at various stages of life may impact the expression of certain diseases, but very little is known about most substances found in foods.

The number of identified, physiologically active phytochemicals has increased dramatically in the past decade. Initial identification of these agents has been made by epidemiological surveys, which indicated the consumption of fruits, vegetables, and grains was correlated with lower incidence of cancer, coronary heart disease, and, more recently, with other diseases. Yet, the correlations frequently do not agree with essential nutrient content. Such results suggest that other food constituents may have physiological activity needed for life and health as well.

## NUTRITION, HEALTH, AND DISEASE

During the past 25 years, epidemiological studies have consistently correlated diet as a factor in the etiology of the five leading causes of death in the U.S., including coronary heart disease, certain types of cancer, stroke, noninsulin dependent diabetes mellitus, and atherosclerosis (Bidlack, 1996). It is

essential to understand the critical role played by food and nutrition in altering the risk for disease (Shils et al., 1999).

Undernutrition still occurs in large groups of people, but nutrient deficiencies, once prevalent, have been replaced in industrialized countries by excesses and imbalances of some food components in the diet. Identification of the external factors that contribute to premature death would aid preventive efforts, improve the quality of life, and reduce health-care costs (McGinnis and Foege, 1993). Even though genetic predisposition increases susceptible people's risk for some of these chronic diseases, these conditions might be diminished or prevented by improvements in the American diet.

## NUTRIENT RECOMMENDATIONS FOR HEALTH

The majority of Americans have heard the message that balance, variety, and moderation are the keys to healthy eating; they also recognize that what they eat may affect their future health (Bidlack, 1996; FNB, NRC, 1989a). The public worries specifically about the food they eat relative to fat and cholesterol. Unfortunately, these same Americans are apt to accept quick fixes from popular health claims.

The "Dietary Guidelines for Americans" were developed by the USDA and DHHS to teach people the fundamentals of proper nutrition. The key recommendations are to choose a diet containing a variety of foods, low in total fat, low in saturated fat and cholesterol, and containing plenty of vegetables, fruits, and grain products; to use sugar and salt (sodium) in moderation; and to maintain a healthy body weight (USDA, 1995). The Committee on Diet and Health, Food and Nutrition Board of the National Research Council (FNB, NRC, 1989a) recommended a decrease in dietary fat intake to 30% or less of total calories. In an effort to promote finite amounts of foods from different food groups, the USDA established the Food Guide Pyramid as a pictorial means to communicate the Dietary Guidelines and the recommended daily allowances (RDAs) (USDA, 1992; FNB, NRC, 1989b).

A review of 200 epidemiological studies by Block and colleagues (1992) indicated that the cancer risk in people consuming diets high in fruits and vegetables was only half that of the population consuming much less of these foods. Populations consuming diets rich in vegetables, fruits, and grain products have been highly correlated with significantly lower rates of cancer of the colon, breast, lung, oral cavity, larynx, esophagus, stomach, bladder, uterine cervix, and pancreas. The strongest support for a protective effect against colon cancer is by fiber-rich foods (Steinmetz and Potter, 1991a,b). Numerous effects of dietary fiber on digestive function are known, although the types of fiber that have positive physiologic response have not been clearly identified. Phytochemicals may also be contributing to the observed protective effects of vegetables. Better health through improved nutrition can increase

quality of life, enhance productivity, maximize the learning potential for each individual, and reduce health-care costs by preventing or delaying the onset of chronic disease.

## BIOACTIVE COMPONENTS AS MEDICINALS

From the beginnings of recorded history, herbs, plants, and specific plant components (leaves, flowers, roots, and bark) have been identified and used in the treatment of specific diseases (Ross, 1998). Even Hippocrates, the father of medicine, included food as a basic part of the treatment to cure disease.

Using modern analytical methods, numerous physiologically active plant constituents have been identified, many of which have been developed into pharmaceutical agents (Cox, 1990). Table 1 identifies 18 well-recognized medicinal agents. These drugs are being used in modern medicine, but perhaps for a different purpose from the use initially described. Structural modification may enhance efficacy and decrease side effects.

Ethnobotany has compared plants used by different cultures from around the world and identified unique biologic (and medicinal) properties. The bioactive components of plants depend on the species, ecology, soil, climate, and growth season of each plant (Cox, 1994). The plants are very sensitive to local conditions and do not always produce the same bioactive chemicals consist-

TABLE 1. Partial list of medicinals derived from ethnobotany.

Drug	Medicinal Use	Plant Source
Aspirin	Analgesic, antiinflammatory	<i>Filipendula ulmaria</i>
Atropine	Pupil dilator	<i>Atropa belladonna</i>
Caffeine	Stimulant	<i>Camellia sinensis</i>
Camphor	Rheumatic pain	<i>Cinnamomum camphora</i>
Codeine	Analgesic, antitussive	<i>Papaver somniferum</i>
Dicoumerol	Antithrombotic	<i>Melilotus officinalis</i>
Digoxin	Atrial fibrillation	<i>Digitalis purpurea</i>
Ephedrine	Bronchodilator	<i>Ephedra sinica</i>
Eugenol	Toothache	<i>Syzygium aromaticum</i>
Pilocarpine	Glaucoma	<i>Pilocarpus jaborandi</i>
Quinine	Malaria propholaxis	<i>Cinchona pubescens</i>
Reserpine	Antihypertensive	<i>R. serpentina</i>
Sennoside	Laxative	<i>Cassia augustifolia</i>
Scopolamine	Motion sickness	<i>Datura stramonium</i>
Tubocurarine	Muscle relaxant	<i>Chondrodendron tomentosum</i>
Tetrahydrocannabinol	Antiemetic	<i>Cannabis sativa</i>
Theophylline	Diuretic, antiasthmatic	<i>Camellia sinensis</i>
Vinblastine	Hodgkin's disease	<i>Catharanthus roseus</i>

ently. These characteristics remain true for the production of current bioactive phytochemicals identified for health protective effects.

As a note of caution, Beecher (1959) reviewed the placebo effects of drugs used for severe pain, anxiety and tension, mood changes, cough, sea-sickness, and the common cold. Thirty-five percent of the patients (1,682) reviewed claimed satisfactory relief of their conditions when given a lactose placebo. This is true of most medical-health relationships between the patient and the practitioner also. Confidence in the practitioner enhances the belief in the therapeutic regimen. Thus, more than one-third of the ethnomedical claims of phytochemical benefits may be due to placebo effects (Farnsworth, 1994).

Herbal remedies have regained popularity with the consumer for self-treatment using natural therapy. In the United States, the most popular herbs include *Echinacea purpurea* L. and other species to enhance immune function, *ginkgo biloba* L. to improve circulation and enhance memory, *Panax ginseng* CA Mey (Asian) and *Panax quinquefolis* L. (American) to increase energy and diminish stress, *Allium sativum* L. (garlic) to reduce atherosclerosis and high blood pressure, and *Hypericum perforatum* L. (St. John's Wort) to relieve mild depression (Karch, 1999; Dewick, 1997). The scientific merit of these therapies and other claims continue to be developed but are currently limited at best.

## NEW HEALTH PARADIGM

A new health paradigm may be evolving that would place emphasis on the positive aspects of diet, identifying components that are physiologically active and that contribute to the prevention of disease onset (Bidlack, 1998). Although not a regulatory category, "functional foods" have arisen as a generic description of the benefits that accompany the ingesting of food for reasons beyond simple nutritional value (Hasler, 1998). Developing bioscience data indicate that diet does modify, perhaps even regulate, numerous body functions related to health. Understanding the mechanisms by which individual nutrients, and non-nutrient constituents, function physiologically should allow food scientists to truly design food products for a healthier diet. Thus, even though genetic predisposition increases people's risk for several chronic diseases, especially associated with advancing age, "optimal nutrition" should enable people to achieve their maximum genetic potential and decrease their susceptibility to disease.

A natural food product can be engineered to become a "functional food" by increasing specific components to reach concentrations more likely to express the beneficial effect, by adding components not normally present but having a beneficial effect, by replacing a component that is excessive and harmful with one having a beneficial effect, or by improving the bioavailability

of components having the desired health benefit (Roberfroid, 1999). The demonstration of such beneficial effects requires a strict scientific approach for which a strategy can be proposed—developing products based on a function-needed approach rather than a product-driven approach. Creation of functional foods as an opportunity to enhance health status rather than promote good or bad foods, or as a marketing gimmick, will make it more acceptable to food and nutritional scientists (Milner, 1999).

## **FUNCTIONAL FOODS**

A growing number of natural products are being promoted as having health benefits; a number of different terms have been used to describe this category, such as functional foods, nutraceuticals, pharmafoods, designer foods, phytochemicals, vita foods, and others. These terms are used in addition to other regulatory categories, such as medical foods, dietary supplements, herbal products, and botanicals.

Initially, the term designer foods was developed by the National Cancer Institute to describe foods that naturally contained, or were enriched with, non-nutritive, biologically active chemical components of plants (phytochemicals) that were potentially effective in reducing cancer risk (Caragay, 1992). The Institute of Medicine of the U.S. National Academy of Sciences, Food and Nutrition Board, defined functional foods as those that encompass potentially healthful products, including any food or food ingredients that may provide a health benefit beyond the traditional nutrients it contains (IOM, NAS, 1994).

In the United States, the functional food category is not recognized as a legally defined entity. The only country that has established a regulatory approval process for functional foods is Japan. There, the category is called Foods for Specified Health Use (FOSHU) with 100 products currently identified and licensed (Arai, 1996).

Nutraceutical was the term first described by the Foundation for Innovation in Medicine (Anonymous, 1991; DeFelice, 1995) to identify any substance considered a food, or part of a food, and provides medical or health benefits including the prevention and treatment of disease. Nutraceuticals may include isolated nutrients, dietary supplements, and diets containing genetically engineered “designer” foods, herbal products, and processed food products such as cereals, soups, and beverages.

The term functional food has been accepted by the food industry and most consumers, and appears to be the best name for the category of physiologically active foods (Bidlack and Wang, 1998). The response of consumers to the different names suggests that “ceutical” reminds people of medicine while “designer” suggests artificial or synthetic. Each of these terms—functional foods, nutraceuticals, designer foods, and medical foods—should be carefully

defined so that consumers and health practitioners do not become confused (Wrick et al., 1993). The significance and relevance of any definition of functional foods depends totally on an adequate description of these foods and substantiation of their health benefits (Head et al., 1996).

The promise of functional foods has emerged at a time when consumer interest in diet and health are at an all-time high (Wrick, 1995). Some of the phytochemicals have shown positive physiological activities in disease prevention, e.g., cancer, heart disease, osteoporosis, and immune response, but most of them will not become silver bullets. More than a dozen classes of biologically active phytochemicals have been identified to diminish cancer and heart disease (Steinmetz and Potter, 1991a, b; Potter and Steinmetz, 1996; Milner, 1997; Craig, 1997). Specific categories to classify the functional properties of phytochemicals are identified in Table 2. Because phytochemicals assigned to these categories may demonstrate more than one bioactive property, the classification may change with further research, e.g., an agent may work well as an antioxidant, but may also inhibit cancer promotion. The actual function may be defined by the physiological concentration found in the food naturally and the amount provided by dietary intake.

## BIOACTIVE PHYTOCHEMICALS

There has been no evolutionary pressure on plants to cause development of food components that would protect man from diseases and cancer; yet, diets rich in fruits and vegetables appear to do just that (Bidlack, 1998). Most likely, these compounds developed as a part of the plant's own defense

TABLE 2. Categorization of functional properties of phytochemicals.

- antioxidants, modifiers of oxidative damage and defense mechanisms related to oxidative stress
- antimutagens, anticarcinogens
- antimicrobial and antiviral bioactive substances
- enhancers of GI function, dietary fibers, probiotics, and prebiotics to alter gastrointestinal functions, and colonic microflora
- immunomodulators stimulate immune function
- anti-inflammatory agents
- cerebroactive, neuroregulatory substances; improve psychological condition
- phytoestrogens
- antihypertensives
- hypocholesterolemic agents
- diminished allergenicity
- antidiabetogenic
- prevention of osteoporosis

mechanisms against environmental insult and only fortuitously provide benefits to man. The challenge to the food industry is to be certain to base its decisions for incorporation of phytochemicals into functional foods on scientifically sound information.

Researchers have examined the chemical constituencies of these foods, isolating and identifying chemical structures and suggesting possible functions for these agents. In many cases, animal experiments have been carried out to test the hypotheses of health benefit and mechanism of action, while, in others, some human testing has been initiated. Some of these compounds may function through multiple mechanisms to enhance health. In addition, the chemical property tested for and identified may not be the biological activity present in the food or in the human body.

Very few physiologically active chemicals have been examined as thoroughly as needed to initiate health claims required by the 1990 National Labeling Education Act (NLEA) and the 1994 Dietary Supplement Health and Education Act (DSHEA) nor to ensure safety from the risk of toxicity and cancer. Bidlack and Wang (1998) identified many of the experiments needed to characterize the physiologically active phytochemicals (Table 3).

The actual health benefits of these phytochemicals, either as natural ingredients, food additives, or as dietary supplements, may not be understood for several years. In addition, the beneficial effects may prove to result from combinations of these chemicals acting by additive or synergistic effects. In all cases, the question of safety must be addressed.

A few of the more well-studied bioactive compounds are listed in Table 4. Some of the phytochemicals that appear to have significant health potential include the following:

- Carotenoids include a family of more than 600 distinct compounds, encompassing the hydrocarbon form, carotene, and the oxygen-containing carotenoid derivatives, xanthophylls.  $\beta$ -Carotene, lycopene, zeaxanthin, and lutein can act as antioxidants, as well as quench singlet oxygen. Epidemiologic evidence correlates carotenoids in vegetables to lower incidence of cancer; however, carotenoids have not been shown to have a direct effect on initiation, promotion, proliferation, or progression of the carcinogenic process. Although a metabolite, retinoic acid does affect proliferation and differentiation—both all-*trans* and 9-*cis* retinoic acid modulate gene expression through unique nuclear receptors (Omaye et al., 1997; Heyman et al., 1992).
- Tea catechins may inhibit initiation and promotion of the carcinogenic processes (Chen, 1992). Using either green tea infusion or isolated tea catechins, these polyphenolic compounds have proven to express a broad spectrum of anticarcinogenic activity in multiple animal models. Importantly, the results of animal experiments consistently indicate the

effective concentrations of these phenolic compounds equal to the levels found in brewed green tea (Yang et al., 1996). Unfortunately, similar results have been harder to verify in the human population.

- Phytoestrogens, such as genestein and daidzein, which are commonly found in soybeans and soyfoods, may decrease osteoporosis, relieve menopausal symptoms, decrease heart disease, and diminish estrogen-enhanced carcinogenesis (Barnes, 1995; Kurzer and Xu, 1997). Theoretically, the phytoestrogens bind to the estrogenic receptor and either compete with or antagonize estradiol action. The beneficial health effect would depend on the exposure level of the phytoestrogen, the binding constant relative to estradiol, and the selectivity of different tissue receptors.
- Tocotrienols exhibit properties different from those of  $\alpha$ -tocopherol (vitamin E). As antioxidants, each of the tocopherols and tocotrienols are effective inhibitors of lipid peroxidation in food and biological systems.  $\gamma$ -Tocotrienol is more effective than  $\alpha$ -tocotrienol in lowering cholesterol synthesis through specific modulation of HMG CoA reductase protein degradation, while  $\alpha$ -tocopherol had no effect. Both  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol effectively inhibit proliferation of cancer cells in tissue culture (Hood, 1998).
- Phenolic derivatives, such as benzoic acid, caffeic acid, catechin, catechol, rutin, vanillic acid, eugenol, and thymol, act as natural antimicrobial agents (Sofos et al., 1998). As natural components of herbs and spices that often provide unique flavoring properties, many of these agents have been used for centuries. These agents protect the public and enhance the shelf life of foods.
- Polyphenols constitute another family of plant compounds that range from simple phenols, such as benzoquinones, phenolic acids, phenylacetic acids, and phenylpropenes to coumarins and isocoumarins, naphthoquinones, and anthraquinones to higher forms such as flavonoids and lignins and to highly polymerized compounds, such as bioflavonoids, proanthocyanidins, or condensed tannins with molecular weights greater than 30,000 Da (Harborne, 1989; Bravo, 1998). Polyphenols have been considered antinutrients, because tannins bind to protein and decrease digestibility (Singleton, 1981; Chung et al., 1998). However, the phenolics have proven to be very good antioxidants, scavenging free radicals and providing metal chelating activities (Shahidi and Wanasundara, 1992). Polyphenols have been implicated in health benefits, such as prevention of cancer and cardiovascular disease.
- Organosulfur compounds found in garlic have antioxidant functions, decrease metabolic activation of carcinogens and adduct binding to DNA, decrease platelet aggregation, and diminish the clotting mechanisms (Chen, 1992; Dorant et al., 1993; Block, 1998).

- Prebiotic enhancement of intestinal function results from stimulation of the growth of beneficial bacteria by fermentable oligosaccharides, such as fructose oligosaccharides and inulin, which produce specific effects on the gastrointestinal physiology, bioavailability of minerals, immune function, colonic tumorigenesis, and regulation of serum cholesterol (Gibson and Roberfroid, 1995; Roberfroid, 1993; Roberfroid, 1996; Roberfroid and Delzenne, 1998; Milner and Roberfroid, 1999).
- Probiotic foods deliver live cultures of microorganisms, such as bifidobacteria, to the gut. The organisms then reculture the gut microflora, contributing positive health benefits, and reducing the risk of colonic disease, non-insulin dependent diabetes, osteoporosis, and cancer (Gibson and Wang, 1994; Naidu et al., 1999). Tomorrow's yogurt may well deliver both an ideal microflora culture and the substrates and activators needed to provide balanced intestinal health.

Only a few examples of physiologically active plant constituents are presented here to represent the exciting potential of some of these agents that may be incorporated into functional foods in the future. A decision based on efficacy must be looked at, identifying the lowest doses needed to produce their physiologic effects, because higher doses may increase the risk for toxicity. In addition, it becomes important to determine if the same dose of the agent in the food has the same efficacy as the isolated compound.

## **REGULATORY ISSUES**

The functional foods concept has brought the medical, nutritional, and food sciences together. Over the past decade, new technologies, such as biotechnology, genetic engineering, food processing, product innovations, and mass production, have enabled food scientists to design new healthful products. Health authorities need to develop new rules and procedures based on rigorous scientific evidence to be effective. A major dilemma of functional foods is that they exist at the interface between foods and drugs (Wrick, 1993). Unfortunately, the existing regulatory system in the United States does not adequately cover this new category of foods.

For regulation, some differentiation is required between those products to be consumed as foods and those products provided from isolates or a concentrated component to be consumed as dietary supplements. Thus, a distinction might be that "functional food" is similar in appearance to conventional food, is consumed as part of a usual diet, and has demonstrated physiologic benefits in reduction of chronic disease beyond basic nutritional functions (Bellisle et al., 1998; Clydesdale, 1997); whereas, a "nutraceutical" is a product produced from foods but sold in pills, powders, potions, and other medicinal forms not

**TABLE 3. Areas of research needed to better characterize the role of the phytochemicals in health.**

- Identify the specific types of photochemicals that provide health benefits
  - determine the strength of epidemiological association
  - characterize the sources, diet or supplements, of phytochemicals that are beneficial or harmful
  - identify the proportion of the population likely to respond positively to phytochemicals
- determine the effective dose of phytochemicals that protect against disease
- determine the concentrations at which pharmacological doses become a toxicological problem
  - evaluate the toxicity of metabolites
- define the effective dose of phytochemicals that provide protection against cancer
  - determine dose response
  - determine effect of intervention on precancerous stage vs. existing tumors
  - evaluate chemical-induced model vs. spontaneous tumor model
  - determine the type of cancer most responsive to specific phytochemicals
  - evaluate timing of dose to the onset of cancer
- identify new mechanisms by which the phytochemicals produce protective effects
- characterize effects of phytochemicals on cell to cell communication
  - determine the effect at various concentrations
  - determine the effect of specific isomers
  - determine specificity relative to lipophilic agents
- determine effects of phytochemicals on cell differentiation
  - determine effect at various concentrations
  - determine effect of specific isomers
  - determine specificity relative to other agents
- determine the effects of phytochemicals on immuno-modulation
  - determine effect at various concentrations
  - determine effect of specific isomers
  - determine specificity relative to other agents
- characterize the factors that affect absorption and bioavailability of the phytochemicals
- determine metabolic fate of absorbed phytochemicals
- identify and characterize metabolites of phytochemical metabolism
- establish the levels of phytochemicals identified with specific tissues
  - determine specific functions of the phytochemicals in these tissues
  - identify the existence of specific binding protein
  - identify selective uptake mechanisms
  - determine species specificity
  - identify differences in metabolic pathways in tissues accumulating different forms
  - determine physiologic activities of metabolic products
- determine optimal phytochemical mixtures
  - determine composition
  - duration of feeding
  - amounts to be fed
- establish the pharmacokinetics of delivered dose
  - evaluate single and combined doses
  - evaluate with and without food sources present
- more closely examine the dietary components associated with health and disease prevention from the diet as a whole

Modified from Bidlack and Wang, 1998.

generally associated with food and has been demonstrated to have a physiologic benefit or provide protection against chronic disease (Scott et al., 1996). Accumulative limits should be set to assure safety, especially if higher intake levels express negative health effects.

## UNITED STATES REGULATIONS

All articles intended for the diagnosis, cure, mitigation, treatment or prevention of disease have been classified as drugs by the Federal Food, Drug, and Cosmetic Act (FDCA, 1938). Thus, the legal display on a label referring to disease prevention or risk reduction associated with consuming a particular functional food is extremely limited at this time. Only four categories are currently identified (Neff and Holman, 1997):

- (1) Ordinary food and nutrients. Health claims can be made on labels only when they are supported by the totality of publicly available scientific evidence, and then only after receiving regulatory approval from the Food and Drug Administration (FDA)
- (2) Dietary supplements. Health claims are based only on "evidence the statement is truthful and not misleading" (Neff and Holman, 1997, p. 28). The FDA must be notified of the statement, and the label must include a disclaimer stating that the agency has not evaluated the claim and that the product is not intended to diagnose, treat, cure, or prevent any disease.
- (3) Medical foods. Health claims must be based on a somewhat higher standard (than for dietary supplements) of being backed by "sound scientific evidence" (Neff and Holman 1997, p. 28). Medical foods do not require FDA approval for claims, though their packages must include disclaimers similar to those of dietary supplements.
- (4) Drugs. Drug claims have the strictest standard. They must be proven safe and effective in FDA-approved and reviewed clinical trials.

## MEDICAL FOODS

In 1988, the orphan drugs amendment to the Federal Food, Drug, and Cosmetic Act provided medical foods with a legal definition:

A food formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation.

Thus, medical foods are complex, formulated products designed to provide complete or supplemental nutritional support to individuals who are unable to ingest adequate amounts of food in a conventional form or to provide

TABLE 4. Bioactive food constituents that may prevent disease.

Active Compounds	Food Source	Potential Health Benefit	Possible Mechanisms and Functions
β-carotene lycopene lutein other carotenoids	tomatoes, carrots, yams, cantaloupe, spinach, sweet potatoes, citrus fruits	reduces coronary heart disease reduces cancer	antioxidant; singlet oxygen and free radical scavenger; induction of cell-cell communication, and growth control; inhibits the proliferation of acute myeloblastic leukemia.
epigallocatechin and epigallocatechin gallate	green tea grapes/wine	reduces cancer reduces heart disease	inhibits initiation, promotion, and progression of cancer. antioxidant, reduces free radical/oxidative damage.
daitzen genestein other isoflavones	soybeans soyfoods	prevents menopausal symptoms prevents osteoporosis reduces cancer	phytoestrogens inhibits the growth of human breast cancer cell lines decrease cholesterol, LDL cholesterol, and triglycerides. stimulates calcium absorption and bone deposition
tocopherols tocotrienols	vegetable oils	antioxidant lowers serum cholesterol inhibits cancer decreases heart disease	inhibits cancer cell proliferation inhibits HMG-CoA reductase
omega-3 fatty acids	fish oil algae flaxseed	reduces serum cholesterol reduces heart disease reduces serum triacylglycerol immunosuppressant	lowers the total and LDL-C:HDL-C ratios. increases serum HDL inhibits arachadonic acid-derived products such as PGE and leukotrienes.
conjugated linoleic acid	dairy products processed vegetable oils	anticancer antiththerosclerosis	inhibits cancer cell growth by interfering with the hormone regulated mitogenic pathway. reduces the LDL cholesterol to HDL cholesterol ratio and total cholesterol to HDL cholesterol ratio in rabbits.

TABLE 4. (continued).

diallyl disulfide and allicin	garlic onions	anticancer stimulates immune function free radical scavenger reduces serum cholesterol reduces serum TG	inhibits the proliferation of human tumor cells in culture. inhibits the metabolic activation of the toxicant and carcinogen. inhibits cholesterol biosynthesis.
sulforaphane and other organic isothiocyanates	cruciferous vegetables	chemoprevention	chemopreventive activity, modulation of drug-metabolizing enzymes.
limonene	citrus fruits	anticancer	regulators of malignant cell proliferation. inhibit post-translational isoprenylation of cell growth-regulatory proteins.
coumarins	vegetables, citrus fruits	prevents blood clotting anticarcinogenic activity	anticoagulants inhibitors and inactivators of carcinogen and mutagen. scavenges superoxide anion radicals.
nondigestible, fermentable oligosaccharides, fructose oligosaccharides	garlic asparagus chicory	intestinal fortification stimulates immune function inhibits tumorigenesis reduces serum cholesterol	prebiotics—effective substrate for bifidobacteria, which are found in the large intestine and are generally considered to be beneficial by stimulating the immune system and protecting body from infection; modulate lipid metabolism.

Modified from Bidlack and Wang, 1998.

specialized nutritional support to patients who have unique physiological and nutritional needs associated with their conditions (Anonymous, 1992). Medical foods differ from the general food supply, because these foods frequently serve as the sole source of nutrition; yet, medical foods are subject to much less scrutiny by the FDA than virtually all other foods categories (DHHS, FDA, 1996). The 1990 National Labeling Education Act (NLEA) specifically exempted medical foods from the NLEA labeling provisions (Yetley and Moore, 1997). There are no specific requirements for label information or substantiation of claims, formulations, and compositional characteristics, manufacturing quality controls, or notification to the FDA of intent to market medical foods.

## NATIONAL LABELING EDUCATION ACT

The 1990 NLEA allows health or disease prevention claims on a food label. A health claim is a statement that expressly, or by implication, characterizes the relationship of any substance to a disease or health-related condition within the context of a total daily diet (DHHS, FDA, 1993). The NLEA requires a prominent panel of nutrition facts, daily reference values, declaration of ingredients, nutrient content, and health claims.

Structure-function claim is a claim on the package that indicates that a nutrient plays a role in a particular biological process, such as "calcium aids in the growth and maintenance of bones" or "high fiber promotes regularity." The claims do not identify a disease entity and cannot refer to treatment, mitigation, or prevention of any disease, disorder, or abnormal physical state. Many countries that do not allow health claims allow structure-function claims, which triggers a declaration of nutrient content.

There were eight original health claims for foods (Table 5) approved by the FDA (DHHS, FDA, 1993 and 1994). A health claim is any claim made on the label that either expressly or through implication (through the use of endorsements, written statements, symbols, or vignettes) characterizes the

TABLE 5. Eight health claims for foods currently approved by the FDA.

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|--|
| (1) Fiber-containing grain products, fruits, and vegetables and a reduced risk of cancer                           |
| (2) Fruits, vegetables, and grain products containing fiber, particularly soluble fiber, and a reduced risk of CHD |
| (3) Fruits and vegetables and a reduced risk of cancer   |
| (4) Calcium and a reduced risk of osteoporosis   |
| (5) Dietary saturated fat and cholesterol and an increased risk of CHD   |
| (6) Dietary fat and an increased risk of cancer  |
| (7) Sodium and an increased risk of hypertension   |
| (8) Sugar alcohols and reduced risk of dental caries   |

relationship between any substance and a disease or health-related condition (DHHS, FDA, 1994). Health claims were derived from the U.S. Dietary Guidelines. An additional claim, "folate prevents neural tube birth defects," has been added since publication of the last dietary guidelines in 1995. In 1997 and 1998, oat soluble fiber and psyllium soluble fiber, respectively, were specifically allowed limited health claim status for foods.

New regulations might be needed to ensure some form of limited patent or copyright on a health claim for companies willing to make the research investment. Otherwise, due to the time delay and costs involved to achieve approval, companies won't bother with investment in clinical trials and will avoid label claims. Although in the long term, a strong claim for health would be very marketable, functional foods may be promoted other ways first. If handled through advertising with satisfied customer statements, only the Federal Trade Commission (FTC) would be involved, and they only require the ad to not be misleading.

## DIETARY SUPPLEMENT HEALTH AND EDUCATION ACT

The 1958 Food Additive Amendments of the Federal Food, Drug, and Cosmetics Act was amended by the Dietary Supplement Health and Education Act of 1994 (DSHEA, 1994). The DSHEA broadly defined a dietary supplement as a product

intended to supplement the diet that bears or contains a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract or combination.

Whereas, a food additive is any substance either intentionally added (direct additive) to food to improve its shelf-life, texture, nutrition, or other aspect of quality, or that unintentionally contaminates (indirect additive) food. Prior to DSHEA, non-nutrient ingredients could have been challenged as unapproved food additives. Under the DSHEA, dietary supplements are exempt from regulations as drugs and for the most part as food additives. The DSHEA now puts the burden of proof for safety of dietary supplements directly on the FDA and limits the agency's authority over their labeling.

The DSHEA did create the Office of Dietary Supplements within the National Institutes of Health to promote and coordinate scientific studies of dietary supplements as they relate to health. In addition, DSHEA mandated formation of the Commission on Dietary Supplement Labels as an independent panel of experts appointed to study and make recommendations about regulating and evaluating label claims and other statements for dietary supplements (Camire and Kantor, 1999). Dietary supplements are classified as food products, but must be labeled as "dietary supplements."

The description of a dietary supplement in the DSHEA specifically includes

- (1) A product that is intended for ingestion in tablet, capsule, liquid, powder, soft gel, or gelcap, or if not in such form, is not represented as conventional food and is not represented for use as a sole item of a meal or of the diet and is labeled as a dietary supplement
- (2) A drug, antibiotic, or biologic, if the product had been marketed as a dietary supplement prior to such approval
- (3) A food, but excludes the definition of food additive
- (4) A food supplement intended to increase the total dietary intake of one or more of the following dietary ingredients: a vitamin, a mineral, an herb or botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake of a concentrate, metabolite, constituent, extract, or any combination of these ingredients (Glinsmann, 1996)

A definition of dietary supplement as broad as this allows for the addition of many ingredients with functional effects that span the food-drug spectrum in terms of use.

With regard to functional foods, DSHEA allows the use of structure/function claims and the dissemination of third-party literature. The 1994 DSHEA provides for statements of nutritional support, which can be applied to a wide variety of dietary ingredients. Specifically, the following claims are allowed:

- to claim a benefit related to a classical nutrient deficiency disease and disclose the prevalence of such a disease in the U.S.
- to describe the role of a nutrient or dietary ingredient intended to affect structure or function in humans
- to characterize the mechanism by which a nutrient or dietary ingredient acts to maintain such structure or function
- to describe the general well-being derived from the consumption of a nutrient or dietary ingredient.

Interestingly, structure/function claims do not require prior approval. The FDA must be notified that such a statement is being made and the following text must be provided by the product label: "This statement has not been evaluated by the FDA. This product is not intended to diagnose, treat, cure, or prevent any disease." The tone of this message may well deter consumer acceptance and thereby diminish industry use, but surprisingly consumers appear to ignore this language. In fact, consumers have stimulated this market because of their individual decision to use products having potential, but mostly unsubstantiated, health benefits.

## INTERNATIONAL REGULATIONS

Differences in international regulations are based on specific differences in the definitions of functional foods and nutraceuticals (Stephen, 1998). In most cases, functional foods reflect use for products in a food form, while nutraceutical reflects use as a pill or concentrate. As mentioned above, the Ministry of Health and Welfare in Japan created FOSHU (foods for specified health use) in 1991 (Arai, 1996). FOSHU products are defined as foods consumed in the normal diet. They are expected to have a specific effect on health due to relevant constituents of foods or are foods from which allergens have been removed. The effect of such addition or removal must be scientifically evaluated to be granted permission to make claims regarding the specific beneficial effect on health expected from their consumption. FOSHU products should not pose a health or hygiene risk.

The Health Protective Branch of Health Canada has proposed a specific definition of functional foods and nutraceuticals. This distinction has been accepted by industry, health professionals, and consumer groups (Stephen, 1998). The Ministry of Agriculture, Fisheries, and Food in the United Kingdom developed a definition to distinguish functional foods and those fortified with vitamins and minerals for nutritional benefits. Similar descriptions are made for functional foods in the European Union (Pascal, 1996). Thus, each regulatory group has identified "functional food" as a food and "nutraceutical" as an isolated form or concentrate.

Concerns within the industry reflect the inconsistency of the regulatory guidelines in the response to functional food products and the lack of direction in promoting the development of products and ingredients that actually can have a positive impact on the health of the consumer. The major regulatory agencies of the world need to adopt a more positive position in regard to certain classes of these products. However, the primary health goal of these agencies must remain the protection of the consumer from harm, including misleading health claims, safety concerns of high concentrations of specific constituents, and potential negative impact on diet diminishing the primary source of nutrients. The consumer must be able to trust that the safety and efficacy controls placed on these health products in turn promotes the quality of the food industry products.

## SAFETY ISSUES

The development of food products to ensure a diet capable of prevention, or treatment, of disease and providing a general health benefit is a relatively new trend. To meet these expanded needs, food companies need a better

understanding of health risk, risk/benefit analysis, evaluation of efficacy and toxicity, and health regulations (Stephen, 1998).

Whether a product is a conventional food or a functional food, all of its additives must have either GRAS (generally recognized as safe) status or FDA approval as an additive. Both health claims and structure/function claims can serve as important mechanisms for manufacturers to convey nutritional benefits to the consumer. These claims must be truthful, not misleading, and supported by sound science. Potential endangerment of the public safety by using unapproved ingredients is not only bad for business but it is against the law (Allen, 1999).

The use of such components in different food products and their suitability for claims in labeling depend on the application of the appropriate standards for safety of use and labeling criteria for a particular product category. In the general food supply, an inherent constituent of the food can be marketed unless it has been found to be "ordinarily injurious to health." As an intentional additive, a functional food component can be used to fortify a processed food, but it requires a stricter condition of "reasonable certainty of no harm" within the context of the total estimated exposure of the "additive." Before DSHEA, a dietary supplement was considered an unapproved food additive in terms of conventional food use because the supplement might be considered adulterated. A functional food component used in a supplement could be less safe than one that occurred naturally or that was intentionally added to a conventional food.

The safety of the functional food component needs to be assessed according to established regulations, including preclinical toxicity tests and pharmacokinetic evaluations of absorption, metabolism, distribution, and excretion. An acceptable daily intake should be determined based on a safety evaluation of exposure derived from historical consumption estimates and the proposed uses in food. Safety of the component should be evaluated under dietary use conditions because the matrix of inherent food components may alter the specific component's bioavailability, metabolism, or mode of action. Toxicity of the bioactive constituent may well be enhanced when removed from that complex natural matrix, suggesting upper limits for intake need to be established (Hathcock, 1995). If so, excessive intakes and pharmacokinetics of the bioactive constituents can lead to toxicity, especially when taken as supplements or concentrates.

Clinical evaluations of food components provide evidence for safety of human exposures, tolerance, and benefit within the context of a total diet. Identification of a specific response can be impeded by lack of an accepted biological marker to serve as an indicator of the effect a nutrient/food component has on a disease or health-related endpoint over time. The use of clinically relevant biomarkers that are well defined in terms of their relationship to a health outcome improves the likelihood that valid conclusions can be deter-

mined (ILSI North America Technical Committee on Food Components for Health Promotion, 1999).

Biomarkers similar to those used in environmental exposures are required for adequate evaluation of the merits and risk of exaggerated intakes of functional foods and constituents (Suk and Collman, 1998; Timbrell, 1998). Biomarkers capable of assessing the following will be required:

- (1) Active agents capable of modifying target tissues (intake biomarkers) require valid intake data and exposures data. To date, these data have not been available due to the questionable reliability of food disappearance data and the lack of information available about most of the functional food constituents of the diet (Ervin and Smicklas-Wright, 1998).
- (2) Specific biological responses that relate directly to either disease risk or health maintenance (effect biomarkers) identify the consequences of interactions between the bioactive food constituent and a specific genomic, biochemical, cellular, or physiologic event. Aimed at predicting a long-term consequence such as general health or disease risk, the use of biomarkers provides a logical scientific basis for major intervention trials, which will, in turn, validate or disprove the biomarkers selected (Halliwell, 1999). Biomarker studies should precede, as well as accompany, major intervention trials that measure disease incidence.
- (3) Modifiers of the response by genetic and other environmental factors (susceptibility biomarkers) may affect the sensitivity of the effect biomarker.

All individuals will not benefit equally from the enhanced intake of specific foods or their bioactive constituents. Understanding these interrelationships will be critical to successfully providing consumers with information about what should and should not be attempted when considering modifications in dietary habits.

It has been increasingly difficult to evaluate the impact of new foods and food products on the well being of society. Testing systems for both toxicity and nutritional quality have become very elaborate, complex, and interrelated, making their interpretation difficult and open to controversy. The issues are no longer those of safety alone, but rather of wholesomeness—Involving the integration of toxicology, nutrition, microbiology, food science, genetics, environmental science, and others (Miller, 1997). “Hazard” and its derivative “risk” are inherent in the biology of the substance and its interactions. With the increased knowledge about the nature of the hazards associated with food, the development of food risk assessment models has grown more difficult. The risk assessment process still remains the best opportunity to provide a reasonable and objective view of the importance of a particular hazard to human welfare. Miller (1997) proposed the development of simplified relative risk models rather than attempting to devise measures of absolute risk. Just

because a product has a positive biologic effect in the body does not automatically guarantee that the product will provide benefit against disease.

For example,  $\beta$ -carotene has been well studied epidemiologically and projected to provide health benefits, acting as an antioxidant and an inhibitor of cancer, if dietary intake was enhanced. Although the hypothesis could still be so, the ATBC study (ATBC Cancer Prevention Study Group, 1994) demonstrated a lack of effect in Finnish smokers who were supplemented, or not, over a six-year period. The results indicated a higher risk in the  $\beta$ -carotene supplemented group. In addition, the CARET study (Omnenn et al., 1996) also demonstrated an increased risk for the supplemented group, and the study was terminated. Recent epidemiologic studies continue to indicate an association between high dietary intake of  $\beta$ -carotene and a lower risk of lung cancer (Cooper et al., 1999). The difference may result from the extreme dosage levels used in the supplements, or the timing of high doses in the clinical trials or a unique protective effect derived from other components in the food matrix when  $\beta$ -carotene is freely available as part of the diet.

A second example provides similar results. Tannins are water-soluble polyphenols present in many plant foods. Many tannin-related polyphenols have been reported to have anticarcinogenic activity, although tannins have also been associated with esophageal cancer in certain regions of the world. Polyphenols also have natural antimicrobial activity for plant protection and may contribute to the regulation of the gastrointestinal microbial population. They are considered nutritionally undesirable because they bind to and precipitate proteins, thereby decreasing their digestibility. Thus, high levels of tannins may cause hepatotoxicity and increase cancer risk, while small quantities may be beneficial (Chung et al., 1998).

National and international food safety legislation needs to be clear, rational, and based on contemporary science, yet be flexible enough to incorporate changes in the scientific base. In addition, legislation must provide for regulations that integrate all components of the food system from production to distribution, provide for adequate enforcement, and deal with all aspects of food safety, including labeling standards. It must unambiguously define authority and responsibility (Miller, 1997). The process of scientific evaluation must be clearly separated from that of policy to assure dispute resolution.

## DESIGNING FUNCTIONAL FOODS

A recent survey of the top 100 food companies identified functional foods/nutraceuticals as the single most important consumer trend impacting new food or food ingredient decisions (Kevin, 1997). As noted above, more consumers believe certain foods and food constituents can provide improved long-term health and decrease their need to use medications. The design and development

of functional foods is a challenge that should rely on basic scientific knowledge relevant to physiologic modulation by food constituents, to assure maintenance of health, and to decrease the risk of disease, while using biomarkers to determine efficacy, while using techniques developed for noninvasive human studies and applicable on a large scale (Roberfroid, 1999).

## GUIDE TO PRODUCT DEVELOPMENT

As a company begins to identify potential products that can fit the functional food category, several questions need to be answered before an investment in time, effort, and financial resources is made (Homsey, 1999).

- What is the health function of the phytochemical to be added? How well has it been researched? Are there multiple functions for the active agent? Are there epidemiological studies to support the claim?
- How much should be used in a food product to ensure delivery of an efficacious amount? How much is found in the food naturally? How much is consumed based on current food consumption data?
- What amount should be added to food to ensure quality? What is the stability of the phytochemical? Potency of the phytochemical may be affected by botanical source, weather, soil conditions, geographic location, processing, and storage conditions.
- What form of the phytochemical is used? An active form, a glycoside, or other natural derivative.
- What effect does the phytochemical have on the end product? Does the ingredient impart changes due to its physical or chemical characteristics—hygroscopic, pH sensitivity, viscosity, cross reaction with other ingredients, color, flavor/odor?
- How stable is the product during storage and shelf life? Is the product affected by light, heat, or oxygen?
- Regulatory issues—does the product fit GRAS or food additive status? Can the product be labeled as a conventional food or as a supplement? Can a structure/function claim be used?
- Will the product still sell even if the health claim does not prove valid?

The answers to these questions will prepare the food scientist with an initial evaluation to establish the validity and the practicality of using the agent in question.

## FUNCTIONAL FOOD PRODUCTS

In 1998, Mazza pulled together information on the nature and physiological effects of biologically active plant components, providing applications of

existing and novel food processing methods to the manufacture of food products with potential health-enhancing properties. Most of the trade magazines continue to promote new products having bioactive ingredients. Examples include the following:

- Ready-to-eat breakfast cereals were one of the earliest functional foods created by W.K. Kellogg, who processed whole grains into a palatable form to deliver fiber and basic nutrients. Today, the cereals are coated with vitamins and minerals, providing a convenient delivery system for better nutrition. In addition, milk and fruit are consumed with these products, enhancing their nutritional value even more.
- Both soluble oat fiber and psyllium fiber have demonstrated contributions to lowering serum cholesterol levels, e.g., incorporation of psyllium into muffin mix, dry and frozen pasta, crisp snacks, cookies, etc. An active ingredient of oat fiber,  $\beta$ -glucan, has also been approved to contribute to lowering of cholesterol.
- Soyfoods are products made from soybeans or isolated soyproteins. The phytoestrogens, genistein and daidzein, are bound to the soyprotein and delivered with soy products. In making tofu, the protein component is precipitated with calcium, providing the food product with a ready source of calcium. Both components contribute to prevention of osteoporosis and postmenopausal problems.
- Designer oils containing eicosapentanoic acid and docosahexanoic acids can be created through selective biotechnology and plant breeding. These oils can be mixed with other oils as ingredients to create specialty health products and can alter the property of that oil for processing as well.
- Modified margarines made from  $\omega$ -3 fatty acids (fish oils, eicosapentanoic acid, and docosahexanoic acid) or plant  $\alpha$ -linoleic acid have been associated with decreased inflammatory activity and a clinical decrease of serum triglycerides. The  $\omega$ -3 fatty acids are actually produced by algae consumed by the fish. To stabilize the oil and decrease the off-flavor, food processors can convert the fatty acids to a margarine product. Margarine is also used as the delivery matrix for products containing phytosterol esters. Two products, "Benecol" and "Take Control," are now on the market. These active constituents can be added to salad dressings as well.
- Grape products, including grape juice, wine, and raisins, deliver several polyphenolic flavonoids such as catechins, anthocyanins, quercetin, and others. These natural delivery systems provide natural antioxidants and anticarcinogens in a very palatable form.
- Breakfast bars/energy bars are generally used to substitute for the

nutrients that would be obtained from the morning meal. High-fiber bars containing nuts and fruit and sweetened by fruit juices are available.

- Vegi Bears are created by fruit and vegetable concentrates, providing a source of phytonutrients.
- Beverages have become all-inclusive liquid delivery systems based on teas and herbal tonics. They are promoted as a mental refresher, including ginkgo biloba and gotu kola, to uplift mood with St. John's Wort, to induce relaxation with chamomile and hawthorn berry, to produce energy with ginseng and guarana, and to provide an immune booster with echinacea and ginseng.
- Herbal snacks include spirulina (algae) snacks, kava kava corn chips, St. John's Wort tortilla chips, puffed rice and corn with spinach concentrate, and others.
- Juices include folate-enriched Tropicana fruit juices and smoothies created with milk and juices, but adding herbal constituents.

The consumer continues to pay for dietary supplements with health-giving properties, for natural foods and health foods, and potentially for enhanced functional foods. The long-term market will depend on how well the consumer is convinced that health benefits are truly being delivered.

## **OPPORTUNITY FOR DEVELOPMENT**

The key change in the new health paradigm is to provide dietary prevention to disease rather than wait until treatment is needed. With the recent economic history of nutritional supplements, natural products, sports drinks, and health foods, all food companies have contemplated, or have already entered into, development of functional foods for their corporate lines.

Consumers are making more health-care decisions for themselves than ever before including lower cost, alternative health care and use of unconventional medical therapies, but not necessarily improving their health. These consumers also select healthy foods that are low in fat, vitamin fortified, and high in fiber. Sales of vitamin supplements and health and natural foods continue to grow. Functional foods, food products and supplements that deliver a physiological benefit in the management or prevention of disease, is a concept that presents an opportunity for future new product growth in the food and beverage industries.

In a recent article, Sloan (1999) noted that the majority of purchase power will result from the aging "Baby Boomers" for the next 30 years. As they turn 50, they comprise a growing component of the total population, approaching 40% by the year 2030, and they hold \$900 billion in purchasing

power. This group has already been attracted to the self-care health movement with exercise and health foods. They will be attracted even more to taking vitamins and minerals to ensure nutritional needs are being met and to products that ensure enhanced performance. The concept of positive eating to contribute to long-term disease prevention has already been embraced.

Using data from the Gallup Survey (1998), Sloan identified 10 up-and-coming nutraceutical markets including the following:

- (1) Joint health—one-third of the U.S. population suffers joint pain and discomfort, in part due to increased exercise. More than half of this group also suffer from various forms of arthritis, which will increase to 60 million Americans by 2020. Natural products claiming to have bioactivity against joint degeneration, inflammation, and pain include gingerol extract,  $\omega$ -3 fatty acids, glucosamine and chondroitin sulfate, and antioxidants.
- (2) Gastrointestinal health—70 million Americans suffer from digestive disorders, 15% of them on a daily basis. One hundred million people suffer from gastroesophageal reflux disease at least once a month, while many others suffer from peptic ulcers, irritable bowel syndrome, gastritis, and constipation, and these problems are expected to continue to increase and affect an additional 35% of the population. Stomach problems are one of the most frequently self-treated ailments and one of the strongest links to herbal remedies, such as ginger, peppermint, fennel, papaya, chamomile, licorice, aloe vera, and others. Increased interest in prebiotic and probiotic products to enhance intestinal health are gaining favor worldwide.
- (3) Blood lipid health—more than 60 million Americans currently have some form of CHD, plaques, and high serum cholesterol levels. Cholesterol-lowering diets are commonly used with pharmaceutic agents. Currently, certain functional foods/nutraceuticals, such as oat bran or psyllium fiber, appear to work, as do  $\omega$ -3 fatty acids and inulin as well.
- (4) Bone density and skeletal strength—33 million Americans (mostly women) suffer from osteoporosis; the majority have already used natural phytoestrogens, inulin, or mineral combinations (Ca, Zn, Mn, and Cu).
- (5) Hormone replenishment—hormones play a critical role in sexual, metabolic, and physical performance, and they diminish with age. Thirty-five million women have menopausal symptoms, while another 10 million have peripausal symptoms. Soy isoflavones and flaxseed have been reported to provide positive response for menopausal symptoms. Male virility appears to be enhanced by androgens and testosterone and may also benefit from arginine or yohimbe intake.
- (6) Body fat—fat deposition can be affected by fat substitutes to decrease dietary calorie loads and fat burners, like herbal phen fen, garcinia cambogia, and Cr, to enhance burning of calories. However, side effects, such

- as noted with phen fen, emphasize the need for clinical testing to identify and minimize potential unexpected side effects.
- (7) Optimal vision—antioxidants have been shown to protect the eye lens against oxidative and light damage that could lead to cataracts, and specific phytochemicals, lutein and zeaxanthin, protect specific regions of the retina.
  - (8) Stress and insomnia—emotional stress and nervousness may be relieved with chamomile, passion flower, and St. John's Wort, and insomnia may be affected by tryptophan or valerian.
  - (9) Breast and prostate health—phytochemicals in fruits and vegetables, grains, and herbals may specifically affect breast and prostate cancer.
  - (10) Gender-specific products are also a hot topic.

Consumers will be looking for foods, beverages, and dietary supplements that help manage or prevent disease, but also enable them to enhance various lifestyle and health conditions (Sloan, 1999). The bioactive plant components listed are derived from herbals and nutraceuticals, as well as functional foods.

"The food industry is interested in functional foods, but not a functional food industry," reported Nancy Childs (Giese and Katz, 1997, p. 58). A defined regulatory pathway for functional foods would increase the likelihood of investment in basic research to support structure-function claims. In addition, to help drive basic and clinical research on functional foods and bioactive ingredients, incentives, such as exclusivity, need to be provided to allow a company to recover its investment through initial capture of market share. It may also mean that corporate partnerships will need to be formed to provide the financial resources to develop these future healthy food products.

## **CONCLUSIONS**

In this century alone, nutrition and food sciences have contributed to the enhanced development of an abundant, nutritious, safe food supply, which has contributed to better health for people around the world (Bidlack and Wang, 1998). Good nutritional status is dependent upon each person having appropriate intakes of micronutrients as well as the expected macronutrients, energy, and access to safe drinking water. Individual nutritional status depends on the availability of sufficient knowledge about appropriate diets, nutrient needs, processing, and food customs to prevent undernutrition and deficiency diseases.

Functional foods represent an interesting challenge for the future of the food industry—an industry that must constantly adjust its products to meet the consumer needs of our ever-changing society—for women's health, increasing mental alertness and well being, and maintaining the physical condition of

the aged population. It is unlikely that a single food component from the thousands that make up our diet will ever be identified as the sole solution to any disease.

The major concerns within the industry reflect the inconsistency of the regulatory guidelines in response to the functional food products and the lack of direction in promoting the development of products and ingredients that actually can have a positive impact on the health of the consumer. The major regulatory agencies of the world may eventually adopt a more positive position in regard to certain classes of these products. The primary role of these agencies remains protection of consumers from harm, including misleading health claims, safety concerns of high concentrations of specific constituents, and potential negative impacts on diet diminishing the primary source of nutrients. The consumer must be able to trust the safety and efficacy controls placed on these health products, which, in turn, promotes the quality of the food industry products.

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