This is to certify that the
thesis entitled

SAFETY AND EFFICACY OF BOTANICAL SUPPLEMENTS

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Horticulture

Major Professor's Signature

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Date

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SAFETY AND EFFICACY OF BOTANICAL SUPPLEMENTS

By

Priyadarshini Raman

A THESIS

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ABSTRACT

SAFETY AND EFFICACY OF BOTANICAL SUPPLEMENTS

By

Priyadarshini Raman

Botanicals have been widely used throughout the world as traditional medicines. Today, several of these botanicals are sold as nutritional or dietary supplements. However, very little scientific research has been done to authenticate the safety and efficacy of these supplements. In this study, we have analyzed over the counter (OTC) supplements such as echinacea, garlic, ginkgo, ginseng, grape seed, kava kava, saw palmetto and St. John's wort for their safety and efficacy. As part of the safety evaluation of these supplements, they were analyzed for the presence of metals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Results indicated that these supplements did not contain unacceptable concentrations of lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium, and tungsten. In addition, analysis for the presence of microorganisms indicated that some of the supplements studied were contaminated with bacteria and fungi. The product claims for most of the supplements suggested that they possess antioxidant and anti-inflammatory activities. The lipid peroxidation and cyclooxygenase (COX) enzymes inhibitory assays conducted on the extracts prepared from the supplements studied revealed that most supplements possessed antioxidant activities. Some of the supplements demonstrated selective COX-1 or COX-2 enzyme inhibitory activities. Therefore, the supplements studied might be useful to prevent or treat inflammatory pain and health problems related to oxidative stress.

To *my beloved parents*

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I dedicate this thesis to my parents, Dr. N. Raman and Mrs. Swarna Raman, for all their love and encouragement, without which my accomplishment would have been impossible. Special thanks goes out to my brother, Gowrishankar Raman, for all his support and patience. I also thank my friend, Ms. Tharakeswari Selvakumar, for her support and help.

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KEY TO ABBREVIATIONS

AD	Alzheimer's Disease
ATSDR	Agency for Toxic Substances and Disease Registry
BHA	Butylated Hydroxy Anisole
BHT	Butylated Hydroxy Toluene
BPH	Benign prostatic hyperplasia
COX	Cyclooxygenase
DMBA	DiMethyl Benz [a] Anthracene
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DPH-PA	3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid
DSHEA	Dietary Supplement Health and Education Act
EDTA	Ethylene diamine tetra acetic acid
EGb	Extract of *Ginkgo biloba*
FAA	Flame Atomic Absorption
GFAA	Graphite Furnace Atomic Absorption
GMPs	Good Manufacturing Practices
GNC	General Nutritional Centers
GSE	Grape seed extract
GSPE	Grape seed proanthocyanidin extract
HAMA	Hamilton Anxiety Scale
HDPE	High Density Poly Ethylene
HEPA	High Efficiency Particulate Air
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hPGHS-2	human prostaglandin H synthase isozyme 2
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
ICP-OES	Inductively Coupled Plasma–Optical Emission Spectrometry
IFN	Interferon
IL	Interleukin

kDa	Kilo Dalton
LDL	Low Density Lipoprotein
LUV	Large Unilamellar Vesicle
MOPS	(3-[N-Morpholino] propane sulfonic acid)
MRL	Minimum Risk Level
MW	Molecular Weight
NaCl	Sodium chloride
NIDDM	Non Insulin Dependent Diabetes Mellitus
NOEAL	No-Observed-Adverse-Effect-Level
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OPCs	Oligomeric ProAnthocyanidins
PAF	Platelet activating factor
PS	Polysaccharide
RDA	Recommended Dietary Allowance
SKT	Syndrome Kurz Test
SLPC	1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine
SPBE	Saw Palmetto Berry Extract
TBHQ	*tert*-butylhydroquinone
TH	T-Helper
TNF	Tumor Necrosis Factor
YMG	Yeast, Malt extract and Glucose

INTRODUCTION

The reliance of human on plants and plant products is unparallel. Ancient history indicates the use of plants and plant decoctions to cure illnesses in addition to its use as food. The documents, many of which are of great antiquity, revealed that plants were used medicinally in China, India, Egypt and Greece. One of the famous surviving remnants is *Papyrus Ebers*, dating back to the sixteenth century B.C. In China, many medicinal plants had been in use since 5000 B.C. The oldest known herbal is *Pen-t'sao* written by emperor Shen Nung around 3000 B.C. Before the advent of modern day medicine, plant derived remedies were the norm in treating illnesses. Therefore, it is understandable why botanicals play an important role in improving overall health.

Most of the medicinally active substances identified in the past were used in the form of crude extracts. The botanical supplements with medicinal claims are now available as easy-to-use formulations, such as tablets, capsules and soft gels. The sale of these botanical supplements has been on the rise in recent years. More people are resorting to botanical supplements for vigor and to maintain vitality of health. In spite of their greater than before usage, very little research has been carried out to ensure the safety of these botanical supplements. The safety of the botanical supplement depends on the raw material, its growing conditions and also on the processing methods. The pesticides used in cultivation can also contaminate the final product. Previous studies indicated relatively high concentrations of metals in botanical dietary supplements. Presence of heavy metals in the diet can be harmful, especially with lead, which causes decreased IQ and poor learning in children. Ingestion of arsenic can cause anemia and leukopenia. There is credible information from epidemiological studies that ingestion of

inorganic arsenic increases the risk of developing skin cancer. Microbial contamination would also be a concern associated with botanical supplements. Therefore, one of the objectives of this study was to evaluate the botanical supplements for the presence of metals and microbes. The study was carried out on eight botanicals, echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort. These botanicals were marketed under the brand names, Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray and Natrol.

Lipid peroxidation is one of the major causes of free radical generation in vivo. It is implicated in many of the chronic diseases. Prevention of free radical generation or its removal can be helpful in maintaining a good health. Cyclooxygenase-2 (COX-2) enzyme is responsible for mediating inflammation and cancer. Inhibitors of COX-2 enzyme are therefore significant in preventing both inflammation and cancer. The supplements studied for food safety, also implicate to support the immune system, help to retain healthy cholesterol levels, healthy cardiovascular function and provide anti-oxidant protection, promote prostate health and enhance the mood. These claims suggest that components present in these supplements possess lipid peroxidation and COX enzymes inhibitory properties. Therefore, the second objective of this study was to test the extracts of these supplements for lipid peroxidation and COX-2 enzyme inhibitory activities in vitro.

This dissertation consists of chapters accounting the details of this research. **Chapter 1** is a literature review, which details traditional uses, chemistry and pharmacological properties of the botanicals studied. It also reviews the current market of the botanical supplements in the US and the reports of metals and microbes present in

them. In **Chapter 2**, the safety aspects of these botanical supplements related to metals and microbes are discussed. The results of in vitro lipid peroxidation and COX-2 enzyme inhibitory properties of the acidic aqueous extract of these supplements are outlined in **Chapter 3**. Chapters 2 and 3 are presented here as manuscripts, each with an introduction, materials and methods, and results and discussion sections.

CHAPTER ONE

LITERATURE REVIEW

General Introduction

Botanicals have been used in the past for the treatment of various ailments. Historically, humans have discovered medicinal plants in their own geographical regions and developed their own recipes and pharmacopoeias. This trend still continues around the world as one of the means to maintain good health. In the United States, the sale of botanical supplements is on the rise. For example, echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort are some of the popular botanical supplements available in the US market. The traditional uses, chemistry and pharmacological properties of these botanicals are outlined in this chapter.

Echinacea

Common names: Purple coneflower, black-sampson, Kansas snakeroot, American coneflower, black susans, comb flower, hedge hog, Indian head, scurvy root (Davis and Cupp, 2000).

Family: Compositae

Species: *Echinacea purpurea, Echinacea angustifolia, Echinacea pallida*

Medicinal parts:

Fresh and dried aerial parts (including flower and flower head), and dried rhizomes and roots of *E. purpurea*, and dried rhizomes and roots of *E. angustifolia* and *E. pallida* are used medicinally (D'Amelio, 1999a; Davis and Cupp, 2000; Tyler, 1993).

Traditional uses:

Echinacea was used in the folklore as a topical application for wounds, burns and insect bites. The roots were chewed for toothache and throat infections as well. In addition, it was administered internally for pain, coughs, stomach cramps and snakebites. The first echinacea preparation, known as Meyers Blood Purifier, was introduced in the market in 1880, for treating rheumatism, neuralgia and rattlesnake bite (Hostettmann, 2003). Lloyd Brothers of Cincinnati, the pharmaceutical company that marketed the echinacea preparation, claimed that the plant was effective for many conditions, including rheumatism, streptococcal erysipelas, stomach upset, migraines, pain, sores, wounds, eczema, sore eyes, snake bites, gangrene, typhoid, diphtheria, rabies, hemorrhoids, dizziness, herbal poisoning, tumors, syphilis, malaria and bee stings (Davis and Cupp, 2000). The introduction of antiinfectives such as the sulfa drugs led the product to fall out of favor (Tyler, 1993).

Echinacea is one of the best known and researched herbs for stimulating the immune system. Many Europeans and Americans use echinacea preparations against colds and flu. In general, the herb is employed for treating common cold, coughs, bronchitis and inflammation of the mouth and the pharynx (Gruenwald et al., 1998).

Chemistry and Biological activity

The chemical constituents of Echinacea species are the polar polysaccharides and glycoproteins, the medium polar caffeic acid derivatives and flavonoids, and the lipophilic alkamides and polyacetylenes (Bauer, 2000).

Studies on the aqueous extract of the aerial parts of *E. purpurea* led to the isolation of two polysaccharides (PS I and PS II) (Stimpel et al., 1984). Structural analysis revealed PS I to be a 4-O-methyl-glucouronoarabinoxylan with an average MW of 35 kDa, while PS II was identified as an acidic arabinorhamnogalactan of MW 45 kDa (Proksch and Wagner, 1987). A xyloglucan (MW 79.5 kDa) was isolated from the leaves and stems of *E. purpurea*. Similarly, pectin-like and arabinogalactan-like polysaccharides were isolated from the expressed juice (Bauer, R., 2000).

Three glycoproteins, with MWs of 17, 21 and 30 kDa, respectively, containing 3% protein, have been isolated from *E. angustifolia* and *E. purpurea* roots. The main sugars were arabinose (64-84%), galactose (1.9-5.3%) and glucosamines (6%) with protein moieties, aspartate, glycin, glutamate and alanin. *E. angustifolia* and *E. purpurea* roots contained similar amounts of glycoproteins, while that of *E. pallida* had less amounts (Bauer, R., 2000).

Caffeic acid derivatives were also a major group of constituents in *Echinacea* species (Figure 1.1). Echinacoside was isolated from the roots of *E. angustifolia* and was the major polar constituent of the roots of *E. angustifolia* (Schenk and Frank, 1996). In *E. pallida*, it occured at a similar concentration and was therefore not suitable for the discrimination of these two species. However, they can be distinguished by the presence of 1,3- and 1,5-O-dicaffeoyl-quinic acids that were present only in the roots of *E. angustifolia* (Bauer et al., 1988a).

The roots of *E. purpurea* lack echinacoside but contain cichoric acid (1,2-Dicaffeoyl-tartaric acid), a compound also present in the aerial parts of *E.purpurea*, *E. pallida* and *E. angustifolia*. In *Echinacea*, cichoric acid occured in high

concentrations in the flower heads (ligules) of the three medicinally used species and in the roots of *E. purpurea* (1.2-3.1% and 0.6-2.1%, respectively). Leaves and stems contained lower amounts of cichoric acid. *E. angustifolia* contained the lowest concentration of cichoric acid. The content of cichoric acid depended on the season and the stage of development of the plant and was highest at the beginning of the vegetation period and decreased during plant growth (Bauer, R., 2000).

The lipophilic constituents of *Echinacea* consisted mainly of alkamides (Figure 1.2), ketoalkenes and ketoalkynes, and essential oil compounds. There were prominent differences in the chemical composition between the roots of *E. angustifolia* and *E. purpurea* (alkamides) and *E. pallida* (ketoalkenes and ketoalkynes).

About 15 alkamides have been identified as major lipophilic constituents of *E. angustifolia* roots (Jacobson, 1967; Bauer et al., 1989). They were mainly derived from undeca- and dodecanoic acid and differ in the degree of unsaturation and the configuration of the double bonds. The main structural type was a 2-monoene-8-10-dienoic acid isobutylamide, but some 2'-methyl-butylamides have also been found (Bauer, R., 2000). In *E. purpurea* roots, 11 alkamides have been identified and in contrast to those of *E. angustifolia*, most of these alkamides possessed a 2,4-diene moiety. Therefore, *E. purpurea* and *E. angustifolia* can be clearly distinguished by their lipophilic constituents (Bauer et al., 1988b).

The aerial parts of all three *Echinacea* species contained alkamides of the type found in *E. purpurea* roots and differed only in the concentration of the constituents (*E. purpurea* > *E. pallida* > *E. angustifolia)* (Bohlmann and Hoffmann, 1983).

The lipophilic constituents of *E. pallida* roots have been identified mainly as ketoalkenes and ketoalkynes with a carbonyl group in the 2-position (Bauer et al., 1988a). They were not abundant in *E. angustifolia* and *E. purpurea* roots and so are suitable as markers for the identification of *E. pallida* roots. But there are no reports of the biological activity of the ketoalkenes and ketoalkynes.

Flowering aerial parts of *E. purpurea* contained less than 0.1% essential oil that consisted of borneol, bornyl acetate, pentadeca-8-en-one, germacrene D, caryophyllene, caryophyllene epoxide and palmitic acid (Bauer, R., 2000). *E. angustifolia* and *E. pallida* contained identical components, and differentiating by the essential oils present, is therefore difficult (Bauer, R., 2000).

Echinacea was reported to increase the non-specific activity of the immune system. Unlike a vaccine, which is active against only a specific virus, echinacea was reported to stimulate immune cells to fight multiple infections (Bauer, 1996). *E. purpurea* was promoted as a phytoimmunostimulant and is being used in the prevention of common colds, coughs, bronchitis, and upper respiratory infections and to treat disorders such as viral infections and chronic disease due to deficiency of immunological responses (Bauer et al., 1999).

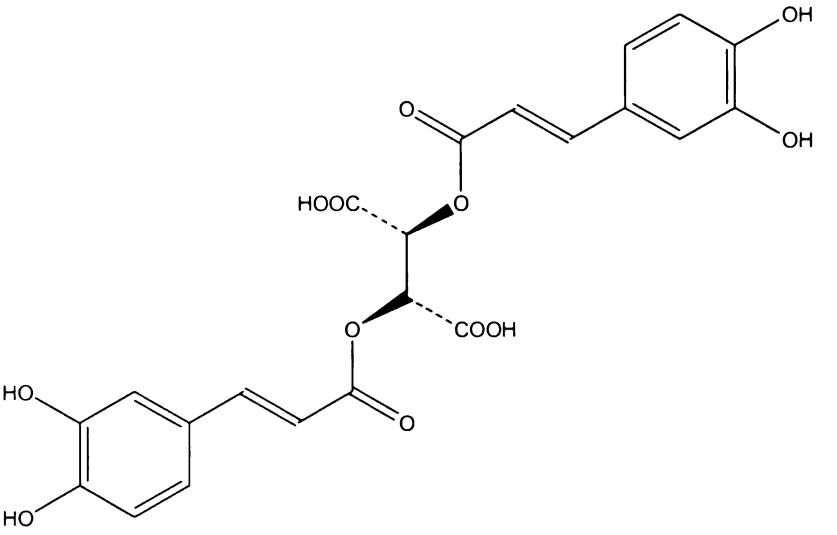
Caffeic acid derivatives have been found to have protective effects on skin connective tissue, in vitro. They were shown to protect collagen from damage caused by the superoxide and hydroxyl radicals generated in a xanthine/xanthine oxidase system. The mechanism of protection was reported to be through scavenging of reactive oxygen species (Facino et al., 1995). Echinacea was found to have antioxidant activities by free

radical scavenging and transition metal chelation and the activity was attributed to the polyphenolic compounds such as cichoric acid and cynarine (Hu and Kitts, 2000).

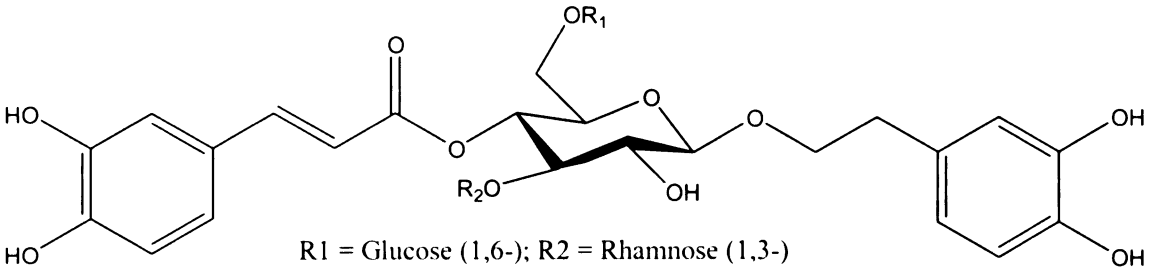
In in vitro experiments, polyunsaturated alkamides from *E.angustifolia* were shown to inhibit microsomal cyclooxygenase and leukocyte 5-lipoxygenase activities, which suggested an anti-inflammatory effect (Muller-Jakic et al., 1994). Anti-inflammatory effect in animals was demonstrated by topical application of the polysaccharide fraction derived from *E. angustifolia* root (Tragni et al., 1985; Tubaro et al., 1987).

Studies in mice using purified polysaccharides from Echinacea plant cell cultures showed a stimulatory effect when applied to immune cells in culture or when injected intraperitoneally into mice. The effects observed were increased phagocytosis, chemotaxis and oxidative burst of either neutrophils or macrophages (Percival, 2000). Purified root extracts containing a glycoprotein-polysaccharide complex exhibited β-cell stimulating activity and induced the release of interleukin 1, TNF and IFN in macrophages (Bodinet and Beusher, 1991). Thus, Echinacea may be regarded as an immunostimulant.

In a study where the leukocytes were isolated from the periphery and then treated with Echinacea extract, it increased neutrophil chemotaxis and bactericidal activity against staphylococcus. Monocytes produced more TNF, IL-6 and IL-1, but not TH_2 cytokines (Roesler et al., 1991). In another ex vivo study, the peripheral blood macrophages were isolated from healthy humans and incubated with freshly pressed *E. purpurea* juice. The macrophages had an increased production of TNF, IL-10, IL-6 and IL-1 (Burger et al., 1997).

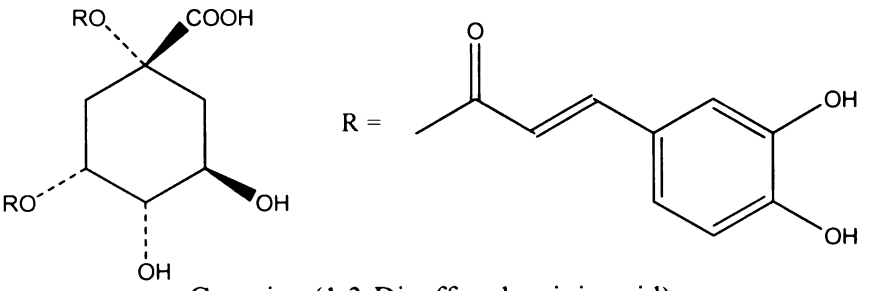


Cichoric acid



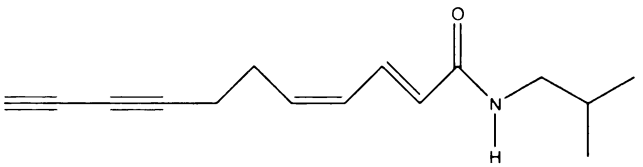
R1 = Glucose (1,6-); R2 = Rhamnose (1,3-)

Echinacoside

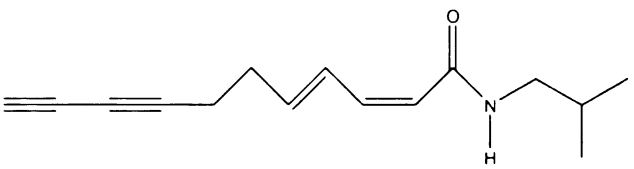


Cynarine (1,3-Dicaffeoyl-quinic acid)

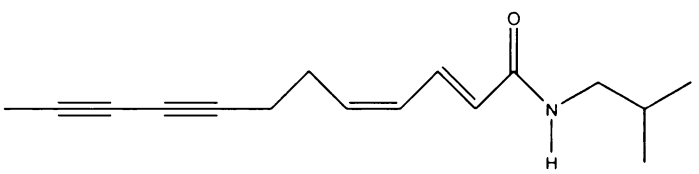
Figure 1.1. Caffeic acid derivatives found in *Echinacea* species



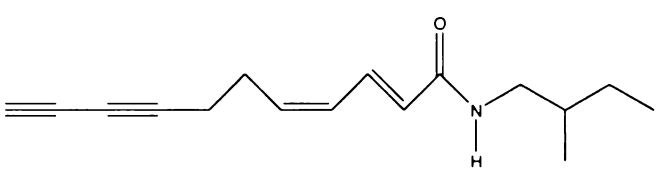
Undeca-2E, 4Z-diene-8, 10-diynoic acid-isobutylamide



Undeca-2Z, 4E-diene-8, 10-diynoic acid-isobutylamide



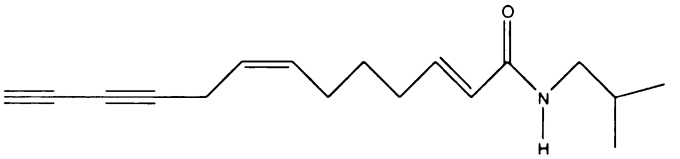
Dodeca-2E, 4Z-diene-8, 10-diynoic acid-isobutylamide



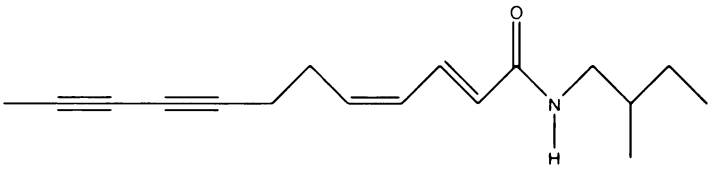
Undeca-2E, 4Z-diene-8, 10-diynoic acid-2-methyl butylamide

Dodeca-2E, 4E, 10E-triene-8-ynoic acid-isobutylamide

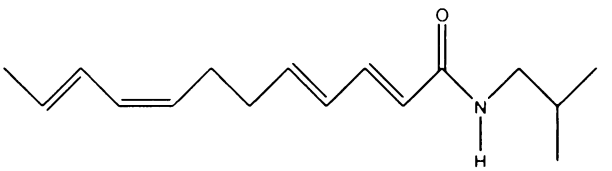
Figure 1.2. Alkamides in *Echinacea* species.



Trideca-2E, 7Z-diene-10, 12-diynoic acid-isobutylamide



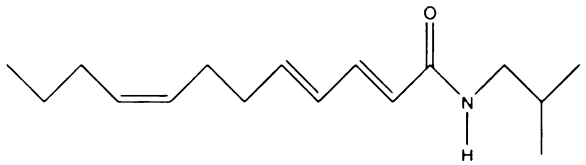
Dodeca-2E, 4Z-diene-8, 10-diynoic acid-2-methylbutylamide



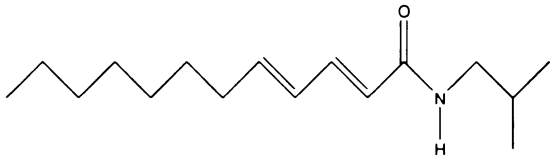
Dodeca-2E, 4E, 8Z, 10E-tetraenoic acid-isobutylamide

Dodeca-2E, 4E, 10Z-tetraenoic acid-isobutylamide

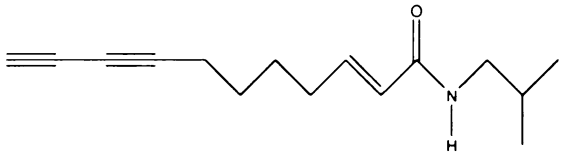
Figure 1.2. (cont'd). Alkamides in *Echinacea* species.



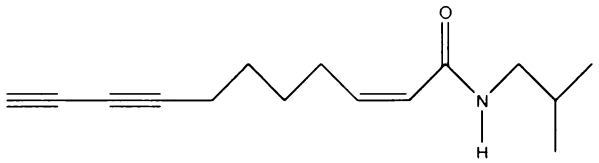
Dodeca-2E, 4E, 8Z-trienoic acid-butylamide



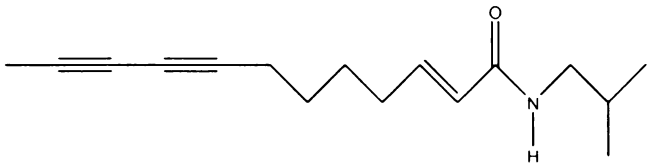
Dodeca-2E, 4E-dienoic acid-isobutylamide



Undeca-2E-ene-8, 10-diynoic acid-butylamide

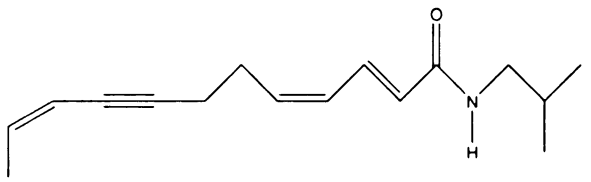


Undeca-2Z-ene-8, 10-diynoic acid-isobutylamide

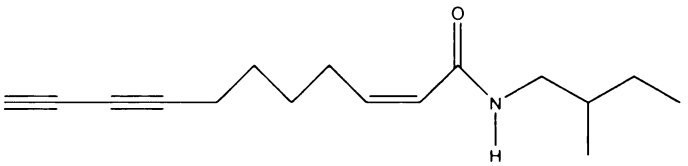


Dodeca-2E-ene-8, 10-diynoic acid-isobutylamide

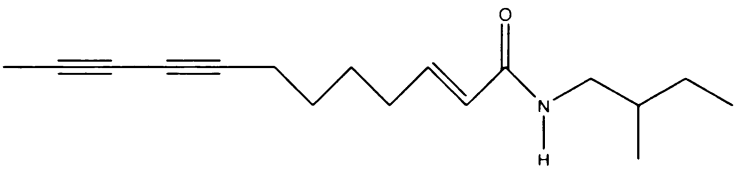
Figure 1.2. (cont'd). Alkamides in *Echinacea* species.



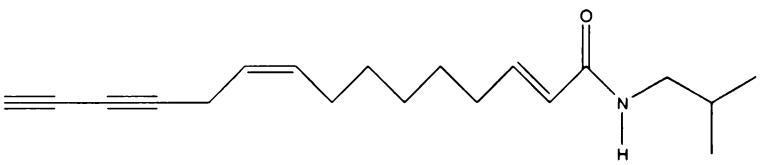
Dodeca-2E, 4Z, 10Z-triene-8-ynoic acid-isobutylamide



Undeca-2Z-ene-8, 10-diynoic acid-2-methylbutylamide



Dodeca-2E-ene-8, 10-diynoic acid-2-methylbutylamide



Pentadeca-2E, 9Z-diene-12, 14-diynoic acid-isobutylamide

Figure 1.2. (cont'd). Alkamides in *Echinacea* species.

Melchart et al. (1995) carried out numerous human clinical trials. Five randomized, placebo-controlled studies involving a total of 134 subjects were summarized. All these studies measured the phagocytic activity of peripheral neutrophils (Melchart et al., 1995). It suggested that Echinacea may be beneficial to those individuals with immune disorders and may show little or no effect on a healthy immune system.

Garlic

Family: Liliaceae

Species: *Allium sativum*

Medicinal parts:

Garlic is used medicinally (D'Amelio, 1999b) and consists of numerous bulblets, which are called "cloves".

Traditional uses:

In ancient Egypt, garlic was a part of the daily diet. It was particularly fed to the working class involved in heavy labor, as in the building of the pyramids. The *Codex Ebers*, authoritative medical text of the era, is one of the earliest sources indicating prescription of garlic for the treatment of abnormal growths, probably representing malignancies of one kind or another. The *Codex* also prescribed garlic for circulatory ailments, general malaise and infestations with insects and parasites.

According to the *Bible*, the Jewish slaves were fed garlic and other allium vegetables, to strengthen them and to increase their productivity. The *Talmud*, a Jewish

religious text, dating from the 2nd century AD, prescribed the consumption of garlic for the treatment of parasitic infection and other disorders.

During the earliest Olympics, it was reported that garlic was fed to the athletes before they competed. Hippocrates, widely regarded as the Father of Medicine, made garlic a part of his therapeutic armamentarium, advocating its use for pulmonary complaints, as a cleansing or purgative agent.

Garlic was widely used in China as part of the daily diet and consumed together with raw meat. In ancient Chinese medicine, garlic was recommended to aid respiration and digestion, most notably diarrhea and worm infestations. In Chinese medicine, a combination of herbs to form a healing tonic is a norm rather than the administration of a single herb. Garlic was frequently used in such combination therapies. Garlic has been linked with the healing process in India from the time of the first available written records. The oldest surviving medical text, *Charaka-Samhita*, suggested the use of garlic in the treatment of heart disease and arthritis.

In 1721, during a pervasive plague in Marseilles, four condemned criminals were recruited to bury the dead. The gravediggers were found to be immune to the disease. Their secret was a concoction they drank consisting of macerated garlic in wine. This was then known as *vinaigre des quatre voleurs* (four thieves' vinegar) and it is still available in France today. Garlic was also believed to assuage constipation when consumed with beverages. Workers outdoors were advised to have garlic to prevent heat stroke (Rivlin, 2001). French priests of the middle ages used garlic to protect themselves against bubonic plague, now established as bacterial infection. During World War I, European soldiers prevented infection by putting garlic directly on their wounds. During

World War II, garlic was known as "Russian penicillin" because it was so effective in treating wound infections when adequate antibiotics were not available (Rivlin, 2001).

Garlic is one of the most extensively studied herbs in natural medicine today. In the United States, garlic is the second most popular herbal supplement. Current research corroborates many of the earlier views concerning the efficacy of garlic in treating many ailments.

Chemistry and Biological activity

Garlic is mainly composed of water (56-68%) and carbohydrates (26-30%). The most important components, medicinally, are the organo-sulfur-containing compounds (11-35 mg/g of fresh garlic) (Nagpurkar et al., 2000). The investigation of garlic usually dealt with the sulfur-containing compounds. This was possibly due to their presence in garlic in high amounts or to the pharmacological activities attributed to various sulfur-containing compounds (e.g., penicillin, probucol).

The mature, intact garlic clove contains mainly cysteine sulfoxides of which the major component is alliin or S-allyl-L-(+)-cysteine sulfoxide (Figure 1.3). The other cysteine sulfoxides are methiin and isoalliin. When garlic is cut, crushed or chewed, the enzyme alliinase is released, converting the cysteine sulfoxides into the thiosulfinates. The thiosulfinates undergo various transformations depending on temperature, pH and solvent conditions, to form more stable compounds such as di- and tri-sulfides, allyl sulfides, vinyl dithiins, ajoenes and mercaptocysteines. The structures of organo-sulfur compounds found in intact garlic and those that are formed during crushing and processing are summarized in Figure 1.4. In specific, alliin, by the action of alliinase, is

converted to pyruvic acid and 2-propene sulfinic acid. The latter is immediately transformed into allicin (Figure 1.3). Air oxidation of alliicin leads to 1,7-dithiaocta-4,5-diene, known as diallyl sulfide (Figure 1.3), the chief constituent of garlic volatile oil (Nagpurkar et al., 2000; Bruneton, 1999a).

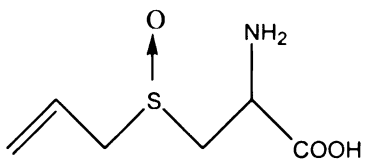
Cardiovascular disease is characterized by factors such as high cholesterol, hypertension, reduced fibrinolysis, increase in blood clotting time and increased platelet aggregation. The cardiovascular protective effects of garlic have been evaluated extensively in the recent years. In animal experiments, feeding of garlic with diet has been demonstrated to lower plasma lipid and cholesterol in rats (Chang and Johnson, 1980; Mathew et al., 1996), rabbits (Bordia and Verma, 1980) and chickens (Qureshi et al., 1983). Number of studies have shown that garlic and garlic preparations significantly reduced plasma lipids, especially total cholesterol and LDL cholesterol in humans (Arora and Arora, 1981; Lau et al, 1987; Steiner et al., 1996). Three meta-analyses of randomized, placebo-controlled human studies confirmed the hypocholesterolemic effects of garlic (Warshafsky et al., 1993, Silagy and Neil, 1994a, Stevinson et al., 2000). Warshafsky et al. (1993) suggested that one half to one clove per day, decreased total serum cholesterol level by about 9%.

Garlic has also been demonstrated to stimulate fibrinolytic activity (Arora et al., 1981; Ernst, 1987). Fibrinolytic activity increased by 72% within 6 h of administration of raw garlic (Chutani and Bordia, 1981). Garlic compounds have been demonstrated to inhibit platelet aggregation (Lawson et al., 1992). The effect of garlic on platelet aggregation in healthy subjects and patients with coronary artery disease was investigated and it was found that long-term administration of a low dosage of garlic led to the

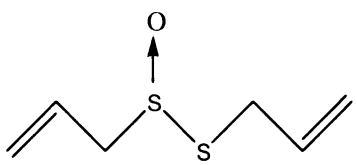
inhibition of platelet aggregation (Bordia et al., 1996). Several garlic compounds have been demonstrated to effectively suppress LDL oxidation in vitro (Lau, 2001). In one human study, subjects who consumed 600 mg tablets of a commercial garlic powder daily for 2 weeks showed reduced oxidation of blood fats by 34% (Phelps and Harris, 1993).

Animal and in vitro studies have provided evidence of the anticarcinogenic potential of several bioactive compounds in garlic (Wargovich et al., 1996). The anticarcinogenic effects of sulfur-containing compounds in garlic, such as diallyl sulfides, have been demonstrated in animals (Reddy et al., 1993). Evidence from available studies suggested a preventive effect of garlic consumption in stomach and colorectal cancers (Fleischauer and Arab, 2001). Garlic has been shown to be a possible biological response modifier and it was reported to reduce the incidence of tumor (Weisberger and Pensky, 1957).

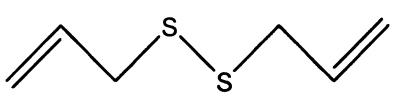
Helicobacterium pylori (*H. pylori*) is a bacterium that has been implicated in the etiology of stomach cancer and ulcers. The incidence of stomach cancer is lower in populations with a high intake of allium vegetables. It has been demonstrated that *H. pylori* is susceptible to an aqueous extract of garlic at a moderate concentration (Sivam, 2001). An aqueous extract of garlic exhibited a broad-spectrum antibiotic activity against gram-positive and gram-negative bacteria (Kabelik and Hejtmankova-Uhrova, 1968). *Enterotoxic coli* strains and other pathogenic intestinal bacteria, responsible for diarrhea in humans and animals, were more easily inhibited by an aqueous extract of garlic than the normal intestinal flora (Caldwell and Danzer, 1988; Rees et al., 1993). Research



Alliin



Allicin



Diallyl disulfide

Figure 1.3. Organo-sulfur compounds found in Garlic cloves.

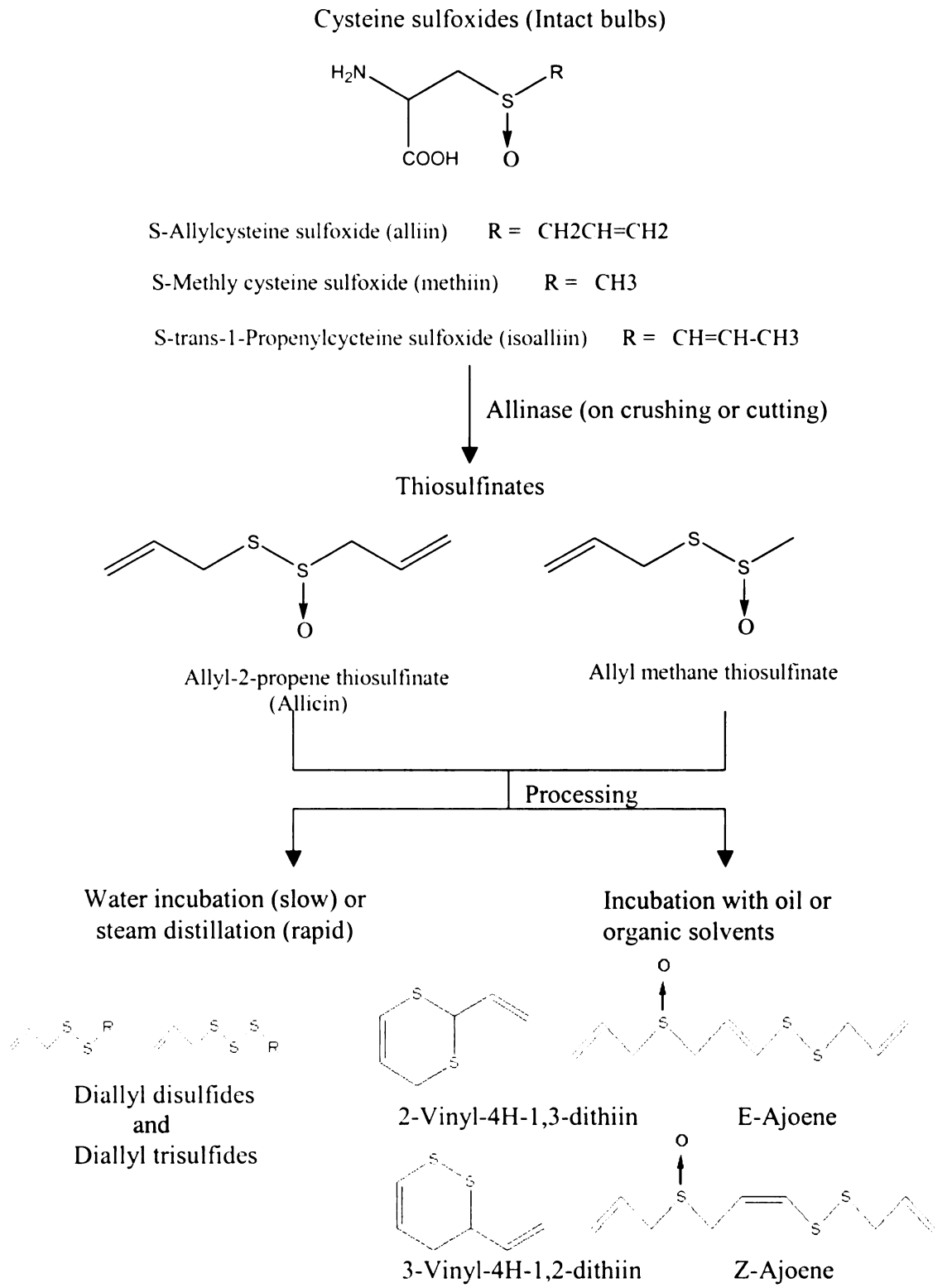


Figure 1.4. Organosulfur compounds present in intact cloves, upon crushing and through processing. Adapted from Mazza and Oomah (2000).

suggested that garlic has profound protective effects against *H. pylori* and other bacterial infections.

Ginkgo

Common names: Maidenhair tree, Flying Moth Leaf, Buddha's Fingernails, Duck-foot, forty-coin tree or *arbre aux quarante écus* (Bruneton, 1999b; D'Amelio, 1999c).

Family: Ginkgoaceae

Species: *Ginkgo biloba*

Medicinal parts:

The leaf extract of the plant is used medicinally today but the seeds were also used in traditional Chinese medicine (Mazza and Oomah, 2000).

Traditional uses:

The ginkgo tree is the only living descendant of many species in the family Ginkgoaceae that flourished more than 200 million years ago when dinosaurs were roaming. It is the oldest known plant and earned the name "the living fossil". The earliest record on the use of *Ginkgo biloba* as medicine dates back to the book of Liu Wen-Tai in 1505 A.D. (Drieu, K., Jaggy, H., 2000). It is described in *Chinese Materia Medica* by Pen Tsao Ching that *G. biloba* was used to treat aging members of the royal society for senility. Although leaf preparations are the primary source of *G. biloba* today, it is the fruits that were described in these ancient Chinese medical records (Strømgaard and Nakanishi, 2004). Ginkgo has been one of the most favored herbs in Chinese medicine for asthma, coughs, allergies, aging, circulatory disorders, and memory

problems. Today, ginkgo nuts are used in Japanese and Chinese cuisine, either grilled or boiled. It was in 1980s when Ginkgo became widely known and used in the United States for medicinal purposes. Millions of Americans and Europeans now enjoy the benefits of ginkgo for memory, cognitive function and circulatory disorders. Ginkgo is the only known circulation enhancer, which can increase blood flow not only to healthy areas of the brain, but also to areas already damaged by disease (Zhang et al., 2000). In addition, ginkgo's powerful antioxidant effects have earned it an international reputation as an "anti-aging" herb.

Chemistry and Biological activity

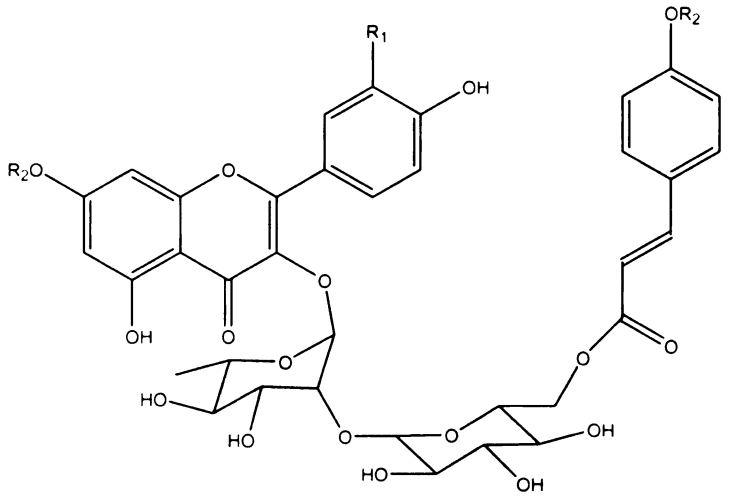
The ginkgo leaf contains two groups of compounds with pharmacological properties: flavonoids and terpenes (diterpenes and sesquiterpenes). The flavonoids are mostly a complex mixture of mono- and diglycosides formed by glucose and rhamnose with kaempferol, quercitin and isorhamnetin as genins (Figure 1.5). Different classes of flavonoids, including dimeric flavonoids, flavonols, flavonol glycosides and coumaric esters of flavonol glycosides have been isolated from *G. biloba* leaves (Mazza and Oomah, 2000).

Ginkgolides are molecules that occur naturally in the leaves and roots of *G. biloba* (Bruneton, 1999b). They have a very specific hexacyclic structure, characterized by a spiro-[4,4]-nonanic sequence, a *tert*-butyl group, and three lactone rings and differ only in the number and positions of substitutes (Figure 1.6). Bilabolide is a sesquiterpene lactone believed to be a degraded ginkgolide (Mazza and Oomah, 2000).

G. biloba extract was prepared by extracting the dried, green leaves with an acetone-water mixture under partial vacuum. After removal of the solvent, the extract was adjusted to a potency of 24% w/w flavonoids and 6% w/w of terpenes (Tyler, 1993).

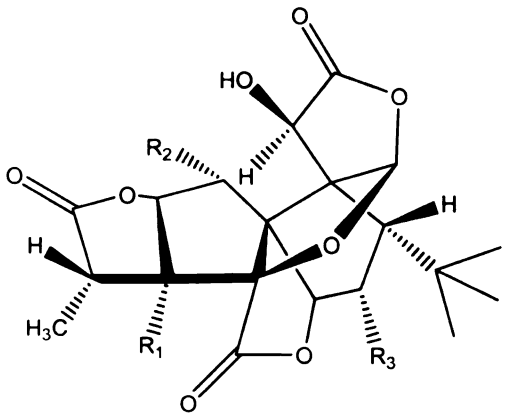
The wide-reaching benefits of ginkgo are thought to be largely due to its effects as an antioxidant, or free radical scavenger. The central nervous system and brain are especially susceptible to free radical damage, and it is believed that gingko's antioxidant action is a major contributor to its "anti-aging" benefits. By preventing free radical damage, ginkgo appears to stabilize cell membranes and render blood vessel walls and red blood cells more flexible, improving the flow of blood and oxygen to the brain, limbs, and other areas supplied by tiny capillaries, such as the eyes and ears. By enhancing microcirculation, ginkgo may improve a variety of brain functions, including memory, concentration, and problem-solving.

The standardized extract of *G. biloba* (EGb 761, 24% *Ginkgo*-flavone glycosides and 6% terpenoids) has been shown to possess neuroprotective properties under conditions like hypoxia, ischemia, seizure activity and peripheral nerve damage (Smith et al., 1996). In an animal study, the performance of mice in an operant conditioning task was used as an index of memory. Using a dose of 100 mg/kg per day, administered orally for 4 to 8 weeks prior to training and then for 10 weeks until a retention test, it was reported that EGb 761 treatment reduced the time to acquisition and enhanced performance on the task, in terms of the number, the effectiveness and the retention of correct responses (Winter, 1991). Ginkgo has been reported to support healthy circulation by inhibiting the effects of a blood clotting substance called platelet-activating factor (PAF) (Smith et al., 1996). The body needs PAF for a number of functions, but



R_1 = H or OH ; R_2 = H or Glc

Figure 1.5. Examples of complex flavonoids from *G. biloba* leaf



Ginkgolide	R_1	R_2	R_3
A	OH	H	H
B	OH	OH	H
C	OH	OH	OH
J	OH	H	OH
M	H	OH	OH

Figure 1.6. Structure of Ginkgolides

excess PAF has been linked to allergies, asthma, inflammatory conditions, and cardiovascular diseases such as stroke.

Ginkgo biloba has been currently recognized as potential cognitive enhancer for the treatment of Alzheimer's disease (AD) (Oken et al., 1998). In a study using neuroblastoma cell line, stably expressing an AD- associated double mutation, it was reported that EGb 761 inhibited formation of amyloid-β fibrils, which are diagnostic and causative feature of AD. It was also noted that EGb 761 decreased the activity of caspase 3, a key enzyme in the apoptosis cell-signalling cascade (Luo et al., 2002). The efficacy of EGb 761 on dementia of the Alzheimer's type was evaluated in a double blind, randomized, placebo-controlled parallel group design in 20 outpatients. The patients were on oral treatment with 240 mg/day of *G. biloba* extract EGb 761 for 3 months. The patients were assessed for attention and memory using SKT test, which is a short cognitive performance test. The results suggested that EGb 761 was effective in mild to moderate dementia (Maurer et al., 1997).

Experimental studies in animals (Karcher et al., 1984) and humans (Schaffler and Reeh, 1985) showed a protective effect of *G. biloba* against tissue damage and impairment of function due to insufficient oxygen, which can trigger excessive oxygen-free radical activity. It was demonstrated that *G. biloba* extract (EGb 761) possessed cardioprotective activity, which was due to the oxygen and nitric oxide free radical scavenging properties (Shen et al., 1996). Another study suggested that the cardioprotective effects were due to the inhibition of free radical formation (Pietri et al., 1997).

Ginseng

Common names:

Panax ginseng: Ginseng, sang, Oriental ginseng, Asian ginseng, Chinese ginseng, Korean ginseng, Ren Shen (Crellin and Philpott, 1990; McGuffin et al., 1997a).

Panax quinquefolium: Ginseng, sang, American ginseng, Redberry, Five Fingers (Crellin and Philpott, 1990; D' Amelio, 1999d).

Panax japonicus: Japanese ginseng, Bamboo ginseng (Bruneton, 1999c; Yun et al., 1998).

Eleutherococcus senticosus: Siberian ginseng, Russian ginseng, sang, eleuthero, Ussurian thorny pepperbush (Crellin and Philpott, 1990; McGuffin et al., 1997b; Kitts, 2000).

Family: Araliaceae

Species: *Panax ginseng*, *Panax quinquefolium*, *Panax japonicus*, *Eleutherococcus senticosus.*

Medicinal parts:

The fresh and the dried roots of *Panax ginseng*, *Panax quinquefolium*, *Panax japonicus*, *Eleutherococcus senticosus* are used medicinally (Yun et al., 1998).

Traditional uses:

The genus name of ginseng "Panax" is derived from the Greek pan (all) akos (cure), meaning "cure-all". This alone tells a lot about this herb: no single herb can be considered a panacea but ginseng comes close to it. Ginseng is one of the most highly revered of ancient Chinese medicinal herbs, for which the references date back to 2600 B.C. *Shen-nung Pent-t'sao Ching*, the first Chinese materia medica written about 2000

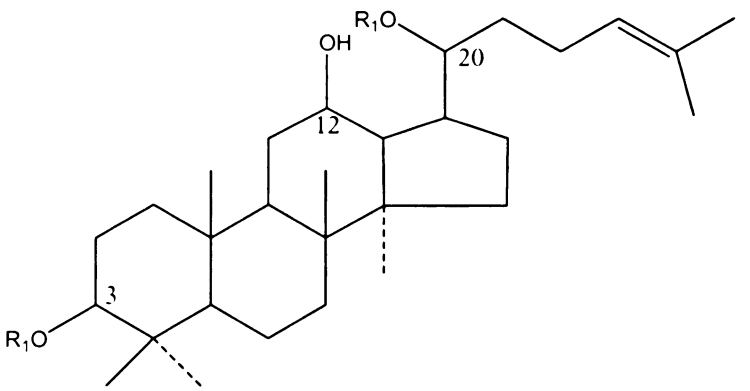
years ago, stated that ginseng was used for its tonic and tranquilizing effects; that ginseng increased alertness, brilliance, and concentration, and improved memory; and that ginseng's prolonged use brought about longevity (Ng and Yeung, 1986). *Panax ginseng* has been used as a general tonic in traditional oriental medicine to increase vitality, health and longevity, especially in older people (Sonnenborn and Propert, 1991). The Chinese name for ginseng, *ren shen*, means 'man-root' for its resemblance to the shape of the human body, with trunk, arms, and legs. While not all of the roots are shaped like the body of a man, those that do are thought to have the power to cure diseases and strengthen both the body and mind. It is used principally in combination with other tonic herbs, as a strengthening tonic alleged to rejuvenate and revitalize the body. Known as Chinese or Korean ginseng, *P. ginseng* is a close relative of American ginseng (*Panax quinquefolium*) (Kitts, 2000). Another herb called *Eleutherococcus senticosus* (Siberian ginseng) is in the same family of Korean ginseng but contained different types of active ingredients (Morgan and Cupp, 2000). It is also classified as an adaptogen and has many of the same clinical applications of the Chinese ginseng. In the western world today, ginseng is commonly considered an "adaptogenic" herb, meaning that it strengthens body functions and the immune system to help people adapt to the effects of physical stress.

P. ginseng is harvested after 2 to 6 years of cultivation and it can be classified into three types based on processing. They are fresh ginseng (less than 4 years old and can be consumed in the fresh state), white ginseng (4 – 6 years old and then dried after peeling), and red ginseng (harvested when 6 years old and then steamed and dried without peeling) (Yun et al., 1998).

Chemistry and Biological activity

The primary bioactive constituents of ginseng are triterpenoid saponins, referred to as ginsenosides. They are present in the root, leaf and berry of the plant. These ginsenosides, attached to various sugar moieties, and flavonoids, are generally considered to be the main bioactive components present in ginseng (Zhang et al., 1979a and 1979b). More than 30 different ginsenoside saponins have been identified and classified into three groups according to the glyco-chain connection on the aglycone backbone. For example, aglycone of 20-S-protopanaxandiol (e.g., ginsenosides Rb1, Rb2, Rc and Rd) are classified as panaxadiol saponins, whereas the aglycone of 20-S-protopanaxatriol (Re, Rf and Rg1) are in the panaxatriol saponin classification. These two groups of ginsenosides are tetracyclic triterpenoid saponins (Figure 1.7 and Figure 1.8). The third group of saponins is oleanolic acid, a pentocyclic triterpenoid. The chemical compositions of individual ginsenosides from Asian and North American ginsengs are very similar except for the different ratio of panaxadiol to panaxatriol of the two species (Hou, 1977). Although more than 30 different ginsenosides have been identified, a group of six ginsenosides, Rb1, Re, Rc, Rd, Rb2 and Rg1, which are named on the basis of individual migration on a thin layer chromatogram, has been chosen as reference standards for ginseng products (Ma et al., 1995). The red ginseng slightly differs in its composition compared to white ginseng (Bruneton, 1999c).

The effects of *P. ginseng* on the quality of life have been studied extensively. In a double blind, placebo-controlled, randomized study, the subjects felt that *P. ginseng* extract improved aspects of mental health and social functioning after 4 weeks of therapy, although these differences assuaged with continued use (Ellis and Reddy, 2002). Another



R1 = R2 = H

20-(s)- protopanaxadiol

Ra : R1 = glucose-6 → 1-glucose-6 → 1-glucose

R2 = glucose-3 → 1-glucose-3 → 1-glucose

Rb1 : R1 = glucose-2 → 1-glucose

R2 = glucose-6 → 1-glucose

Rb2 : R1 = glucose-2 → 1-glucose

R2 = glucose-6 → 1-arabinose (pyr)

Rb3 : R1 = glucose-2 → 1-glucose

R2 = glucose-6 → 1-xylose

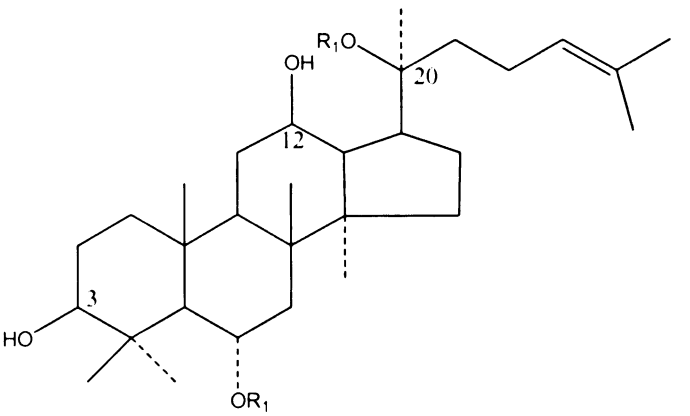
Rc : R1 = glucose-2 → 1-glucose

R2 = glucose-6 → 1-arabinose (fur)

Rd : R1 = glucose-2 → 1-glucose

R2 = glucose

Figure 1.7. Structures of protopanaxadiols.



R1 = R2 = H

20-(s)-protopanaxatriol

Re : R1 = glucose-2 → 1-rhamnose

R2 = glucose

Rf : R1 = glucose-2 → 1- glucose

R2 = H

Rg1 : R1 = glucose

R2 = glucose

Rg2 : R1 = glucose-2 → 1-glucose

R2 = H

Figure 1.8. Structures of protopanaxatriols.

double-blind, placebo- controlled, randomized clinical trial claimed that chronic ginseng supplementation – at either its clinically recommended level or twice that level – did not enhance mood in healthy young adults (Cardinal and Engels, 2001).

Ginsenosides were demonstrated to protect against myocardial ischemia/reperfusion damage with simultaneous reduction in lipid peroxidation. It was proposed that the cardiovascular protection by the ginsenosides may be partly mediated by the release of nitric oxide, a potent antioxidant (Chen, 1996). It was also reported that a standardized extract of ginseng reduced lipid peroxidation by 15% as measured by malondialdehyde levels and was also effective in reducing injuries and inflammation produced by eccentric muscle contractions (Cabral de Oliveira et al., 2001).

Red ginseng extract, administered at 50 – 400 mg/kg, inhibited DMBA/Croton oil-induced skin papilloma in mice, decreased the incidence of papilloma, prolonged the latent period of tumor occurrence and reduced tumor number per mouse in a dose-dependent manner. This result suggested that red ginseng extracts possessed therapeutic activity and might improve the cell immune system (Xiaoguang et al., 1998). In a case-control study, it was noted that there was a decrease in the risk of human cancers with increasing frequency and duration of ginseng intake (Yun and Choi, 1995).

The effect of ginseng on newly diagnosed Non Insulin Dependent Diabetes Mellitus (NIDDM) patients has been evaluated. In a double-blind, placebo-controlled study, 36 NIDDM patients were administered with ginseng (100 or 200 mg) or placebo for 8 weeks. Ginseng therapy was reported to elevate mood, improve psychophysical performance and reduce fasting blood glucose and body weight. The placebo was found to reduce body weight and to alter the serum lipid profile but there was no change in

fasting blood glucose. It was suggested that ginseng may be a useful therapeutic adjunct in the management of NIDDM (Sotaniemi et al., 1995). Ginseng therapy has also been evaluated for its efficacy as an effective alternative in erectile dysfunction (Choi et al., 1995; Hong et al., 2002).

Grape seed

Family: Vitaceae

Species: *Vitis vinifera*

Traditional uses:

The medicinal and nutritional values of grapes (Vitis vinifera) have been heralded for thousands of years. Ancient Greek philosophers praised the healing power of grapes - usually in the form of wine. European folk healers developed an ointment from the sap of grapevines to cure skin and eye diseases. Grape leaves were used to stop bleeding, inflammation, and pain, such as the kind brought on by hemorrhoids. Unripe grapes were used to treat sore throats and dried grapes (raisins) were used in constipation, and thirst. Grapes were used to treat a range of health problems including cancer, cholera, smallpox, nausea, eye infections, and skin, kidney, and liver diseases (Bombardelli and Morazzoni, 1995).

Grape seed extract is one of the primary commercial sources of natural antioxidants. They are collagen-protective pigments called oligomeric proanthocyanidins (OPCs). OPCs and related phenolics are also found in berries, blackcurrant, green tea, black tea, and many other plants. There are no traditional uses of OPCs. However, berries, grapes, and other food sources have been perceived as generally healthful.

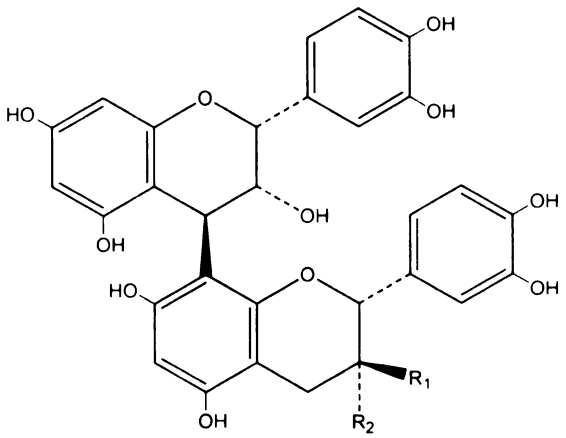
Chemistry and Biological activity:

Oligomers or polymers of catechin and epicatechin are present in abundant amount in seeds of grapes (Bombardelli and Morazzoni, 1995). These compounds are also referred to as procyanidins or leucoanthocyanins. The procyanidins are constituted by a number of flavan units regularly linked by C4-C6 or C4-C8 bonds. The simplest procyanidins are dimeric, but trimers, tetramers and oligomers up to 8 units may be present in the procyanidin mixture isolated from grapes. The procyanidins B1-B4 characterized by 4 → 8 linkage, are the most common dimers, sometimes accompanied by corresponding 4 → 6 linked isomers (Figure 1.9). In addition, catechin monomers are also present in great abundance (Katalinić, 1999). Apart from proanthocyanidins, grape seed extract has several compounds but OPCs have been attributed for the broad array of biological effects.

Proanthocyanidins have been reported to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Afanas'ev et al., 1989; Buening, et al., 1981; Kolodziej et al., 1995). Further more, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation and capillary permeability and fragility and to modulate the activity of enzyme systems including cyclooxygenase and lipooxygenase (Bors and Saran, 1987; Kolodziej et al., 1995).

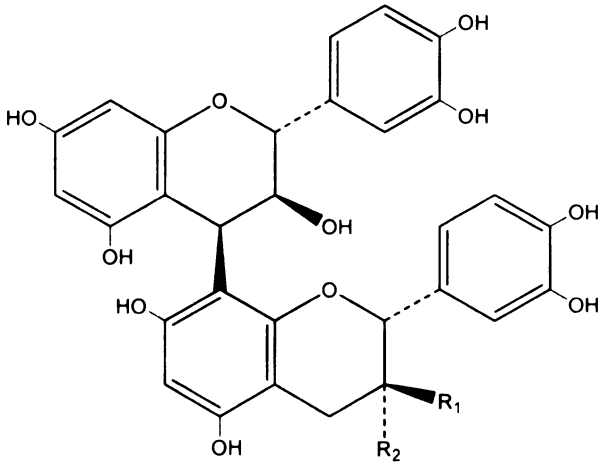
Grape seed extract (GSE), rich in polyphenols (proanthocyanidin), is manufactured by extracting grape seeds with aqueous ethanol. Various proanthocyanidins obtained from grape seeds were tested for their scavenging capacity for superoxide and hydroxyl radicals in aqueous models and were found to be potent free

radical scavengers (Ricardo da Silva et al., 1991). Another study involving H_2O_2/NaOH/DMSO system also demonstrated the free radical scavenging activity of Grape seed extract (Yamaguchi et al., 1999). In an animal study, which compared grape seed proanthocyanidin extract (GSPE) to vitamin C, vitamin E succinate and beta-carotene, GSPE showed significantly higher antioxidant activity (Bagchi et al., 1998). This antioxidant activity was proposed to prevent the progression of cataract formation (Yamakoshi et al., 2002). GSE has been demonstrated to exert anticancer effects against various human carcinoma cells in culture (Agarwal et al., 2000a and 2000b; Ye et al., 1999). It has been shown to strongly inhibit the growth of human prostate carcinoma DU145 cells in addition to apoptotic cell death in culture (Agarwal et al., 2000a) and in nude mice (Agarwal et al., 2002).



Procyanidin B_1 $R_1 = OH, R_2 = H$

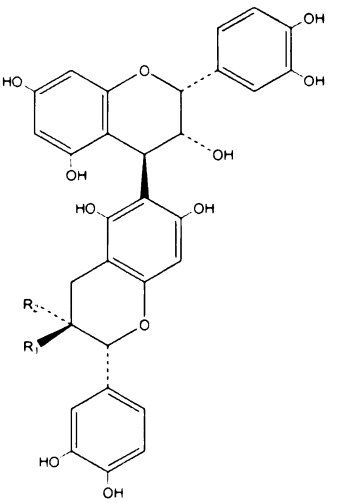
Procyanidin B_2 $R_1 = H, R_2 = OH$



Procyanidin B_3 $R_1 = OH, R_2 = H$

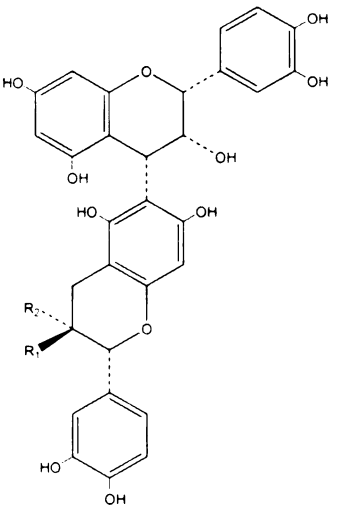
Procyanidin B_4 $R_1 = H, R_2 = OH$

Figure 1.9. Structures of the main procyanidin dimers from *V. vinifera*.



Procyanidin B_5 $R_1 = H, R_2 = OH$

Procyanidin B_7 $R_1 = OH, R_2 = H$



Procyanidin B_6 $R_1 = OH, R_2 = H$

Procyanidin B_8 $R_1 = H, R_2 = OH$

Figure 1.9. (cont'd). Structures of the main procyanidin dimers from *V. vinifera.*

Kava-kava

Common names: Kava pepper, awa, kew, tonga, kawa, yaqona, sakau, ava, ava pepper, intoxicating pepper (Reeder and Cupp, 2000; McGuffin et al., 1997).

Family: Piperaceae

Species: *Piper methysticum*

Medicinal parts:

The dried roots of the plant are used medicinally (Reeder and Cupp, 2000).

Traditional uses:

Kava-kava is consumed as an intoxicating beverage usually prepared from the roots of the kava plant *Piper methysticum*, in the islands of the South Pacific. Traditionally, kava-kava extracts were prepared from macerated roots with water and coconut milk (Norton and Ruze, 1994). The beverage causes a tranquil state of intoxication. These extracts have been consumed over the last 2000 years without any harmful effects on health (Steiner, 2000). The Westerners have sought after the intoxicating effects of the beverage, as a beneficial alternative to alcohol in reducing anxiety and as a therapy for sleeplessness and menopausal symptoms. Several commercial preparations, such as capsules, tinctures and fluid extracts, have been available in Europe and the USA. Recently, the safety of kava-kava products is in question due to the reports on hepatotoxic side effects. There have been 24 cases of severe liver damage reported, including three requiring transplants and one death from the use of standardized extracts containing 30-70% of kava lactones (Denham et al., 2002). Epidemiological studies in the Northern territories of Australia did not show liver

damage in a population where traditional extracts of kava have been consumed by individuals regularly in quantities 10 – 15 times the recommended daily dose of kava lactones. In the traditional preparations of the kava root, the kava lactones are balanced by the availability of glutathione in the preparation. In the available preparations of the standardized extract that relate to hepatotoxicity, only the kava lactones have been present in the products and no additional glutathione was taken along with the product. This difference in the glutathione levels would explain the differences in toxicity (Whitton et al., 2003).

Chemistry and Biological activity:

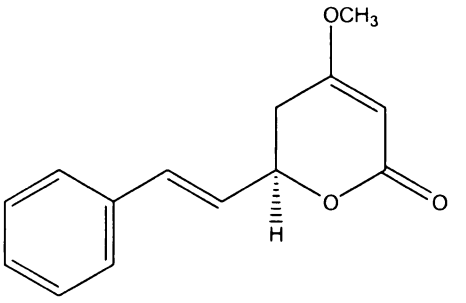
The active ingredients of kava-kava belong to a family of styrylpyrones called "kavapyrones" or "kavalactones". A total of 18 kavalactones have been identified at present and kawain, yangonin and dihydromethysticin (Figure 1.10) are the predominant pharmacologically active components among them (He et al., 1997). The remaining kavalactones are derivatives of kawain, yangonin, or dihydromethysticin (Bruneton, 1999e).

Kava-kava has been shown to be effective as an alternative treatment in anxiety. Clinical studies have shown that kava lactones were effective in the treatment of anxiety at subclinical and clinical levels, anxiety associated with menopause and anxiety due to various medical conditions (Singh and Singh, 2002). An extract of kava kava, WS 1490, was administered to 101 outpatients who were suffering from anxiety of non-psychotic origin in a 25-week, multicenter, randomized, placebo-controlled, double-blind trial. The

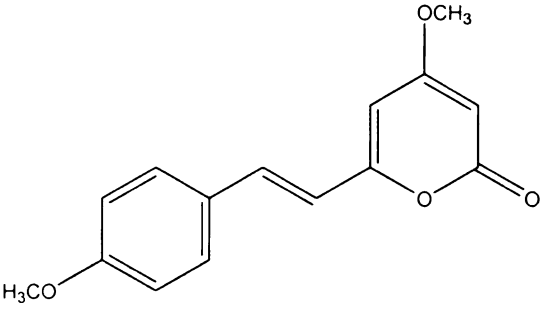
outcome based on the Hamilton Anxiety Scale (HAMA) confirmed the advantage of the kava extract with little or no adverse effects (Volz and Kieser, 1997). A meta-analysis of seven clinical studies also validated that kava kava was more effective than placebos for anxiety treatment (Pittler and Ernst, 2000).

In a study of women with hot flashes, sleep disturbances, or emotional problems related to menopause, the women who received kava kava were found to have reduced symptoms in all three categories as compared to those receiving a placebo (Warnecke and Gynakologe, 1991). Kava kava possibly worked as a mild painkiller. This belief was supported by animal studies using kava kava extracts to reduce pain sensitivity. Four different substances in kava kava were found to individually reduce pain. The mechanism of action was thought to be different from that of opiates and may be of interest to persons who do not tolerate codeine (Jamieson and Duffield, 1990). A cancer incidence study in the Pacific Islands indicated that higher kava consumption lowered the cancer incidence (Steiner, 2000).

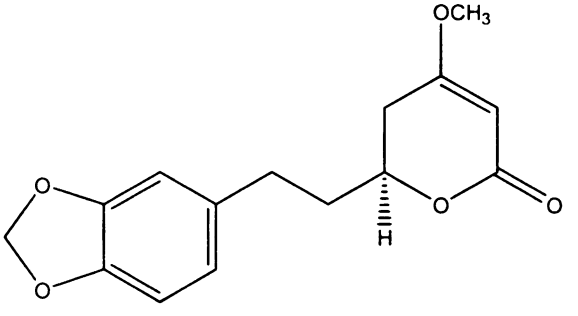
Long-term use of kava at high doses is associated with flaky, dry, and yellowish discoloring of the skin; ataxia; hair loss; partial loss of hearing; loss of appetite; and body weight reduction. The dermatologic signs of excessive kava use are known as *kava dermopathy* or *kavaism* (Norton and Ruze, 1994) and they are usually reversible on discontinuation of use (Jappe et al, 1998). Kavaism has thus far been observed only in the inhabitants of the South Pacific, who regularly ingest doses at least 100 times higher than those recommended for therapeutic use (Ernst, 2002).



Kawain



Yangonin



Dihydromethysticin

Figure 1.10. Examples of kavapyrones from *P. methysticum* (kava-kava).

Saw palmetto

Common names: Palmetto, Dark palmetto, Fan palm, Sabal, dwarf American palm (D' Amelio, 1999f; McGuffin, 1997d; Bruneton, 1999d).

Family: Palmaceae

Species: *Serenoa serralata*

Medicinal parts:

The berries of the plant, which are brownish-black to bluish-black, and somewhat oily, are used medicinally (D' Amelio, 1999f).

Traditional Uses:

Saw palmetto is a dwarf palm tree that grows in Texas, Florida, Georgia, and southern South Carolina. The plant produces purple-black berries from September to January. The earliest known use of saw palmetto was in the 15th century BC in Egypt to treat urethral obstruction. The Native Americans also used saw palmetto to treat genitourinary conditions. In the early 20th century, it was used in conventional medicine as a mild diuretic and as a treatment for benign prostatic hyperplasia (BPH) and chronic cystitis. Historically, saw palmetto has also been used to increase sperm production, increase breast size, and increase sexual vigor. Early settlers in the United States observed that animals, which ate the berries, grew fat and healthy, and by the 1870s saw palmetto was purported to improve general health, reproductive health, disposition, and body weight, and to stimulate appetite (Meadows and Cupp, 2000).

Chemistry and Biological activity:

The components of Saw palmetto that have received the most attention are the lipids. The oil is made up of triacylglycerides with fatty acids of chain lengths usually less than 14 carbons. They are predominantly lauric, myristic and oleic acids (Bone, 1998). The berries also contain flavonoids, terpenoids and polysaccharides. There are remarkably no unusual alkanes, alkenes, polyprenols, sterols or free fatty acids in the nonpolar extracts. β -Sitosterol was the major sterol reported in the fruit (Bombardelli and Morazzoni, 1997). The hexane soluble fraction is used in the symptomatic treatment of Benign Prostatic Hyperplasia (BPH) (Diamond and Towers, 2000).

Various studies have demonstrated that saw palmetto reduced symptoms associated with benign prostatic hyperplasia. Its efficacy was similar to medications like finasteride. However, saw palmetto was better tolerated and less expensive (Gordon and Shaughnessy, 2003). The proliferation of a set of prostatic derived cell lines: 267B-1, BRFF-41T and LNCaP, was examined using Saw Palmetto Berry Extract (SPBE) as neat oil. The proliferation was inhibited randomly when dosed for 3 days with SPBE. It was also observed to inhibit COX-2 expression, an enzyme associated with an increased incidence of prostate cancer (Goldman et al., 2001). In a randomized, double-blind, placebo-controlled trial of saw palmetto, 85 men with lower urinary tract symptoms were administered saw palmetto or placebo for 6 months. It was observed that saw palmetto led to a statistically significant improvement in urinary symptoms in men with lower urinary symptoms compared with placebo (Gerber et al., 2001).

St. John's Wort

Common names: Goat weed, klamath weed, rosin rose, amber touch and heal, tipton weed, John's wort, hypericum, iperico (Schwarz and Cupp, 2000).

Family: Hypericaceae

Species: *Hypericum perforatum*

Medicinal parts:

The herb (leaves and stem) and the flowering tops of the plant are used medicinally (McGuffin et al., 1997c; D'Amelio, 1999e).

Traditional uses:

Hypericum is a perennial aromatic shrub with bright yellow flowers that bloom from June to September. The flowers are said to be at their brightest and most abundant around June 24th, the day traditionally believed to be the birthday of John the Baptist. The name of the plant, perhaps originated from St. John's Day. The plant is native to Europe and can also be found in the United States and Canada. It grows in dry fields, roadsides, and woods.

Historically, St. John's wort has been used to treat neurologic and psychiatric disturbances (anxiety, insomnia, bed-wetting, irritability, migraine, excitability, exhaustion, fibrositis, hysteria, neuralgia, and sciatica), gastritis, gout, hemorrhage, pulmonary disorders, and rheumatism, and has been used as a diuretic. Some forms of the herb have been used topically as an astringent and to treat blisters, burns, cuts, hemorrhoids, inflammation, insect bites, itching, redness, sunburn, and wounds (Schwarz

and Cupp, 2000). Though it had been used for a plethora of indications, in more recent time it has found its place in the treatment of depression and anxiety disorders.

Chemistry and Biological activity:

St. John's wort extract is a chemically complex material with a large number of bioactive components. The bioactive agents in St. John's wort are phloroglucinols, anthracene derivatives, flavonoids and essential oils (Mazza and Oomah, 2000). The phloroglucinols consists predominantly of the prenylated derivatives: hyperforin (Figure 1.11) and adhyperforin (Brondz et al., 1983). The anthracene derivatives consist of the naphthodianthrones, which include hypericin (Figure 1.12), pseudohypericin, isohypericin, and their chemical precursor proto-hypericin and hypericodehydrodianthrone (Upton, 1997). Flavonoids constitute about 11.7% of the leaves and 7.4% of the stalks (Upton, 1997). These include kaempferol, luteolin, myricetin, quercitin and the flavonol glycosides hyperoside (hyperin), quercitrin, isoquercitrin, amentoflavone, luteolin and rutin. Essential oils constitute about 0.06-0.35% of the plant. The major components of the essential oil are 2-methyl octane, α-pinene, β-pinene, limonene, myrcene, caryophyllene and humulene (Mazza and Oomah, 2000).

Several studies have demonstrated the effectiveness of St. John's wort in treating depression. In a randomized, double-blind, placebo-controlled, multicenter study, the clinical efficacy of St. John's wort demonstrated that it was effective in treating subjects with mild to moderate depression and the therapeutic effect depended on its hyperforin content (Laakmann et al., 1998). Many studies have corroborated its superior efficacy to

placebo and comparable efficacy to standard antidepressants in the treatment of mild-to-moderate depression (Nathan, 1999; Barnes et al., 2001). Two meta-analyses of clinical trials have also concluded the effectiveness of St. John's wort in treating mild to moderate depression (Linde et al., 1996; Kim et al., 1999). Though St. John's wort has been demonstrated to be effective in treating mild to moderate depression, it was not effective to treat major depression (Shelton, 2001). The potentially serious adverse effects of St. John's wort monotherapy were photosensitization and induction of manic symptoms in predisposed patients (Schulz, 2000). Problems may also arise when patients take St. John's wort with other medications as it induces a hepatic enzyme through activation of the cytochrome P450 system. Thus St. John's wort can decrease the plasma level of a large number of prescribed drugs (Ernst, 2002).

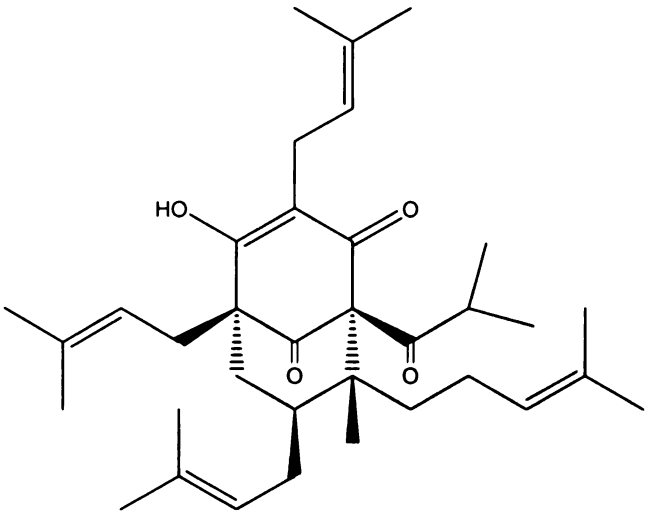
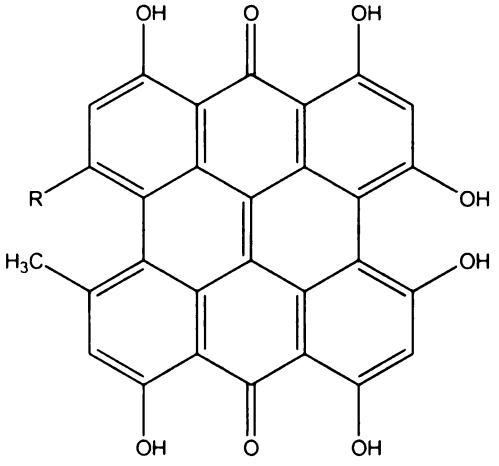


Figure. 1.11. Structure of Hyperforin.



Hypericin R= CH_3

Pseudohypericin R= CH_2OH

Figure 1.12. Structure of Hypericin and Pseudohypericin.

Food Safety aspects of botanical supplements

The sale of the botanical supplements is on the rise in the USA. A recent survey showed that 12 to 37% of US consumers have used herbal medicines (Eisenberg et al., 1998). The awareness of the people to maintain good health, and maintain vitality and vigor could be one of the reasons for the increasing sale of these supplements. However, very little research has been carried out to ensure the safety of these products.

Earlier studies on the concentrations of heavy metals present in the botanical supplements indicated that relatively high concentration of metals were present in them. Huggett et al. (2001) studied the concentrations of metals such as chromium, nickel, arsenic, cadmium and lead in Valerian, St. John's wort, Passion flower and Echinacea supplements. Chromium, nickel, lead and arsenic were detected in concentrations less than or equal to 25 ng/g. But, cadmium was detected at higher concentrations in all samples studied (less than or equal to 967 ng/g). In another study of 95 dietary supplements for arsenic, cadmium, mercury and lead, it was reported that 11 of the products exceeded the tolerable intakes of the population such as children, women of childbearing age, and pregnant women (Dolan et al., 2003).

Another study reported the presence of toxic levels of lead, arsenic and mercury in patented Chinese patent medicines (extracts of herbals in the form of pills, tablets and/or liquids) sold in the United States (Au et al., 2000). There were also reports of toxic heavy metals in Asian and Chinese herbal medicines (Kang-Yum and Oransky, 1992; Wong et al., 1993; Espinoza et al., 1996; Ernst et al., 2002). In addition to heavy metals, presence of microorganisms can also pose health hazards to the consumers. It was reported that *Aspergillus flavus* were present in 11 out of 62 medicinal plants tested

(Halt, 1998). Aflatoxin was reported to be present in traditional herbal drugs from India and Srilanka (Roy et al., 1988; Abeywickrama and Bean, 1991).

These studies indicated that more research is to be directed towards the safety of the botanical supplements in respect to metals and microbes present in them. Therefore, we have analyzed some supplements for the concentrations of metals present in them using Inductively Coupled Plasma – Mass Spectrometry. We have also analyzed these selected supplements for the presence of bacteria and fungi.

The literature review of the botanicals studied indicated that some of them possessed cyclooxygenase enzyme and lipid peroxidation inhibitory activities. The supplements label also suggested that they possess cyclooxygenase enzyme and lipid peroxidation inhibitory activities. Therefore, we have analyzed some of these supplements for inhibition of cyclooxygenase enzymes and lipid peroxidation in vitro. The results of the studies will provide information regarding the safety and efficacy of the supplements, which provide the consumers with needed efficacy and safety data.

CHAPTER TWO

EVALUATION OF METAL AND MICROBIAL CONTAMINATION IN BOTANICAL SUPPLEMENTS

Abstract

The sale of botanical dietary supplements in the USA is on the rise. However, limited studies have been conducted on the safety of these supplements. There are reports on the presence of undesired metals in some of the botanical dietary supplements. In our study, we have analyzed echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort supplements manufactured by Nature's Way, Meijer, GNC, Nutrilite, Solaray, Sundown and Natrol, for lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium, and tungsten using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Results indicated that the botanical supplements analyzed did not contain unacceptable concentrations of these metals. We have also evaluated these supplements for the presence of microbes and found bacteria and fungi in some of these supplements. Bacteria were present in Nature's Way Korean ginseng, Meijer garlic, GNC garlic, Solaray garlic, Natrol kava kava, Sundown Korean ginseng, Sundown echinacea and Sundown garlic. Fungi were present in Nature's Way echinacea, Solaray St. John's wort, Natrol echinacea and Sundown saw palmetto.

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Introduction

The market for botanical dietary supplements in the United States of America has increased over the past years (Eisenberg et al., 1998). Some consumers depend on botanical dietary supplements to maintain mental acuity and to overcome problems associated with aging such as benign prostatic hypertrophy, elevated blood pressure and cholesterol levels, and effects of menopause. Others resort to dietary supplements for energy, endurance and to relieve stress. Other reasons for the popularity of dietary supplements are the higher health care costs and the desire for a healthy living. However, very little is known about the safety of these supplements.

The Dietary Supplement Health and Education Act (DSHEA) of 1994 defines dietary supplement as "a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: vitamins; minerals; herbs or other botanicals; amino acids; dietary substances for use by man to supplement the diet by increasing the total dietary intake; or concentrates, metabolites, constituents, extracts, or combinations of these ingredients" (Pub. L., 1994; U.S.C.). The safety of the dietary supplement is dependent on the growing conditions of the raw material and its extraction, formulation and manufacturing processes. The pesticides used in the cultivation of botanicals might contaminate the dietary supplements as well (Khan et al., 2001). Earlier studies indicated the occurrence of relatively high concentrations of metals in botanical dietary supplements (Khan et al., 2001; Hight et al., 1993; Huggett et al., 2001; Chuang et al., 2000; Wong et al., 1993; Au et al., 2000; Ernst, 2002; Moore and Adler, 2000; Dolan et al., 2003).

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) is one of the fastest growing techniques for trace element analyses because it enables rapid multielement

determinations at the ultra-trace level. Although other techniques such as Flame Atomic Absorption (FAA), Graphite Furnace Atomic Absorption (GFAA) and Inductively Coupled Plasma–Optical Emission Spectrometry (ICP-OES) are efficient to determine low levels of elements, ICP-MS is superior mainly due to multielement capabilities, speed of analyses, low detection limits and isotopic capabilities. This technique has been successfully used in the analysis of plant samples (Dolan et al., 2003; Dombovari et al., 2000; Hokura et al., 2000; Koplik et al., 1999; Krachler et al., 2002; Rodushkin, 1998; Leiterer et al., 1997; McCurdy, 1990).

Echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort were few botanicals among the top twenty selling herbals in 1999 (Blumenthal, 1999). We have analyzed these popular botanical dietary supplements for the presence of metals using ICP-MS. The metals quantified included lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium and tungsten.

Microbial contamination is a concern associated with food products. Improper handling and storage of dietary supplements can result in microbial contamination. Therefore, we have also analyzed these supplements for microbial contamination.

Materials And Methods

Inductively Coupled Plasma-Mass Spectrometry

The samples were analyzed using an Inductively Coupled Plasma-Mass Spectrometer (Micromass® Platform ICP-MS) using a Meinhard concentric nebulizer as the sample introduction system. The ICP-MS spectra were scanned for a period of 1.5 min. Before data acquisition, the ICP-MS was optimized with a standard solution that contained 10 μg/L of Be, Co, In, Ce, Bi, and U. Parameters such as torch position and

gas flow rates were adjusted until the maximum and most stable signal was observed for the wide range of masses.

The concentrations were calculated based on linear regression techniques using a series of standard solutions spiked with 40 ppm Calcium. The elements indium and bismuth were used as the internal standards. Standards that were within 15% of the expected concentrations were used to determine the calibration lines. The concentrations of standards ranged from 0.05 to 100 µg/L for ^{51}V, ^{52}Cr, ^{75}As, ^{98}Mo, ^{105}Pd, ^{114}Cd, ^{120}Sn, ^{121}Sb, ^{184}W, ^{205}Tl, ^{208}Pb, ^{238}U and 0.5 to 1000 µg/L for ^{63}Cu and ^{66}Zn, to ensure that unknown samples were within the range of the standards.

A Barnstead Thermolyne Corp. Nanopure® Infinity Ultrapure water purification system (Model no: D8961) was used for the preparation of the reagents. Standard solutions for calibration and internal standard solutions were prepared from commercial single element analyte standard solutions (Spex/Fisher Scientific). Optima nitric acid (A467-1, Fisher Scientific) was used for the preparation of calibration solutions and for sample digestion. Reagent grade hydrochloric acid (A508SK-212, Fisher Scientific) was used for cleaning the Teflon® vials and storage bottles.

Botanical dietary supplements, echinacea, garlic, ginkgo biloba, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort, sold under the brand names such as Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray and Natrol, were procured in 2002 and 2003 from stores in Michigan, Illinois and Indiana.

Sample preparation and metal analyses were conducted in class 100 clean rooms. Teflon® vials (0103L, Savillex) used for digestion of samples and Nalgene HDPE sample bottles (03-313-2A, Fisher Scientific) used for the storage of digested sample were rinsed three times with deionized water. They were then filled with hydrochloric acid solution

(15%) and capped. The vials and bottles were then placed in a water bath (45°C). After 24 h, the hydrochloric acid was emptied and the vials and bottles were rinsed with deionized water. They were then put in a tub containing deionized water. After 24 h, they were removed and dried under a class 100 HEPA- filtered laminar airflow hood.

Sample Preparation

The supplements analyzed were in the form of capsules, tablets or soft gels. For capsules, the shells were removed, contents emptied into an agate mortar and ground well. Tablets were also made into a fine powder using agate mortar and pestle. The soft gel capsules were weighed and digested and each soft gel was considered as one analytical portion. The powdered samples were weighed (approximately 400mg) in acid washed Teflon vials and capped. Ten milliliters of Optima nitric acid were added to the vials, sonicated for 2 h and then left at room temperature for 4 h. The vials were then placed on the hot plate (approximately 75°C) for 16 h. Once the solutions were clear, 6 mL of Optima Nitric acid were added to each vial and placed on the hotplate (approximately 75°C) for 24 h. The vials were then sonicated for 2 h, 6 mL of Optima nitric acid was added and placed on the hot plate (approximately 75°C) for 18 h. The solution from the vials was evaporated, 10 mL of 10 M Optima nitric acid were added to the vials and the resulting solution was stored at room temperature in 30 mL Nalgene HDPE sample bottles till analyses (adapted from Scelfo et al., 2000).

Quantification of metals

A full mass scan (m/z ratio ranging from 7 to 240) of solutions prepared from supplements were carried out to determine the range of elements present in it beyond the background threshold. An example of a full mass scan is presented in Appendix 1. The metals present in the samples were determined by comparing the data obtained from full

mass scan to the relative abundances of naturally occurring isotopes. The results from the preliminary analysis indicated that lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium, and tungsten were present in detectable concentrations. We, therefore, chose to quantify these metals in the dietary supplements studied.

Preliminary analyses indicated that most supplements contained a high concentration of Calcium and hence to match the matrix present in the samples, 40 mg/L of calcium were added to all the calibration standard solutions. The solution analyzed in the ICP-MS had 1 mL of sample solution, 1 mL of 2% Optima nitric acid, and 2 mL of a solution of 20 μg/L of In and Bi, used as internal standards. The total daily intake of each metal was calculated based on the recommended dose of the particular supplement (Tables 2.2-2.9).

Determination of microbial contamination

The bottles containing the dietary supplements were wiped with 70 % ethanol under aseptic conditions in a laminar flow hood. The bottles were opened under sterile conditions and 1 unit of the sample was transferred into a test tube containing 5 mL of physiological saline solution (1 unit of sample refers to 1 capsule / 1 tablet / 1 soft gel). The mixture was vortexed and then kept in the laminar flow hood for 30 min. The solutions/suspensions were then vortexed again and an aliquot of 100μL lawned on YMG plates (Yeast, Malt extract and Dextrose media) and incubated for 3 to 14 days at 28°C. The plates were monitored for the growth of bacteria or fungi.

Results And Discussion

The concentrations of metals present in the supplements studied were compared with the Minimum Risk Level (MRL), the No-Observed-Adverse-Effect-Level (NOAEL)

or the Recommended Dietary Allowance (RDA) for each element. The MRL and the NOAEL values are defined by the Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services (ATSDR, 2004). MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse noncancer health effects over a specified duration of exposure. MRLs are based largely on toxicological studies in animals and on reports of human occupational exposure. The given MRL is based on an average body mass of 70 kg. The NOAEL is defined as the dose of a chemical at which there are no statistically or biologically significant increases in frequency or severity of adverse effects between the exposed population and its appropriate control. Effects may be produced at this dose, but they are not considered to be adverse (ATSDR, 2004). Recommended Dietary Allowance (RDA) is listed for the metal when MRL/NOAEL values are not defined. RDA is the average daily dietary intake level that is sufficient to meet the nutritional requirements of nearly all healthy individuals in a particular life stage and gender group (Introduction to Dietary Reference Intake, 2002). The MRLs /NOAELs/ RDA of the metals analyzed in the supplements are listed in Table 2.1.

Concentrations (μg/day) of lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium and tungsten quantified using ICP-MS in the supplements studied are listed in Tables 2.2 - 2.9. The results were compared with MRLs/NOAELs for each metal and found that all supplements studied contained less than the minimum risk levels of above metals for adults. The minimum risk levels for palladium, antimony, thallium and tungsten are not available.

Because of the widespread use of lead in plumbing and painting materials, its intoxication is a concern to both children and adults. The human brain is most affected by lead doses. Children appear to be especially sensitive to lead because of a greater accessibility to lead in the nervous system of the young. Lead exposure was correlated to decreased IQ and poor learning in children (Reichlmayr-Lais and Kirchgessner, 1997).

Our results indicate that lead concentration in supplements analyzed did not pose health risk including pregnant women and children, since the tolerable intake levels for lead for adult and pregnant women are 75 and 25 μg/day, respectively. We also analyzed the supplements for uniformity between batches under the same brand names. One batch of Echinacea by Sundown had 7.37 μg of lead whereas the samples from other two batches of the same product gave 0.24 and 0.30 μg of lead, respectively. This result indicated that batches of a product of the same brand might have botanicals sourced or grown under different environments.

The concentrations of cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum and tin were found to be less than the respective MRL/ NOAEL/ RDA values in all the botanical supplements studied. There were no reference data available in the literature for palladium, antimony, thallium and tungsten. But their concentrations were low in most supplements. However, tungsten was present in ginkgo (Meijer), saw palmetto (GNC) and St. John's wort (GNC) at 5.33, 8.77 and 36.01 μg/day, respectively.

Zinc, copper and molybdenum are considered to be good for human health. Zinc acts as a catalyst, coactive or structural unit for some enzymes (Johnson, 1997). Cuproenzymes, in which copper acts as cofactor, are essential for the normal functioning of the body. Molybdenum is a component of the sulfite oxidase enzyme. This molybdoenzyme catalyzes the last step in the pathway of degradation of sulfur amino

acids (Johnson, 1997). Zinc, Copper, Molybdenum were present in high concentrations in all supplements analyzed.

Assessment of the botanical supplements for the presence of microorganisms

"Microorganisms" means yeasts, molds, bacteria and viruses and includes, but is not limited to, species having public health significance. The term "undesirable microorganisms" includes those microorganisms that are of public health significance, which may subject a dietary product to accelerated decomposition.

Among the botanical supplements analyzed for the presence of microorganisms, 13 samples tested positive for bacteria and fungi (Table 2.10). The results indicated the presence of bacteria and fungi in echinacea by Nature's Way. Bacteria were present in Nature's Way Korean ginseng, Meijer garlic, GNC garlic, Solaray garlic, Natrol kava kava, Sundown Korean ginseng, Sundown echinacea and Sundown garlic. Fungi were present in Nature's Way echinacea, Solaray St. John's wort, Natrol echinacea and Sundown saw palmetto. One batch of GNC garlic was not contaminated whereas two other batches were contaminated with bacteria.

Microbial contamination in botanical supplements may result from production conditions and could decompose the supplement during storage. Microbial contamination can also occur due to improper handling of the material during production and packaging. Botanical supplements tainted with microorganisms could pose serious health risk to consumers. Therefore, further research is to be directed towards the identification of the type of microorganisms present in the dietary supplements. Typing the organisms can be helpful in distinguishing the microorganisms that will be of public health significance from the ones that are not harmful to human health.

Good Manufacturing Practices (GMPs) should prevent the presence of all microorganisms including undesirable microorganisms. Sourcing of the raw materials is also of great importance in improving the safety of the supplements. In conclusion, manufactures of botanical supplements should emphasis and adhere to safety standards applicable to food processing in addition to efficacy and dosage.

Table 2.1. Minimum Risk Levels / No-Observed-Adverse-Effect-Levels / Recommended Daily Allowance of the elements

Metal	MRL/ NOAEL/RDA per day	Reference
Lead	75 µg for adults, 25 µg for pregnant women, 6 µg for children	Carrington and Bolger, 1992
Cadmium	14 µg*	ATSDR, 1999a
Arsenic	21 µg*	ATSDR, 2000
Uranium	140 µg*	ATSDR, 1999b
Chromium	35µg for males and 25µg for females‡	Chromium, 2002
Vanadium	210 µg*	ATSDR, 1992
Copper	10 mg†	Copper, 2002
Zinc	21 mg*	ATSDR, 2003a
Molybdenum	63 mg†	Molybdenum, 2002
Tin	21 mg*	ATSDR, 2003b

* MRL

† NOAEL

‡ RDA

Table 2.2. Concentrations of metals determined in Echinacea supplements by ICP-MS. The concentrations are represented in μg/day.

	Echinacea						
Metals	Nature's Way	Meijer	GNC	Nutrilite	Sundown	Solaray	Natrol
Lead	0.567	0.440	0.927	0.093	0.710	2.901	0.034
Cadmium	0.071	0.077	0.096	0.029	0.049	0.967	0.004
Arsenic	0.434	0.137	0.793	0.150	0.235	0.908	0.027
Uranium	0.133	0.024	0.744	0.095	0.064	0.173	0.002
Chromium	8.838	2.033	9.374	4.340	4.516	4.562	0.125
Vanadium	3.292	1.020	6.769	0.751	7.047	7.025	0.022
Copper	34.715	17.389	10.428	12.684	14.404	33.353	1.302
Zinc	38.761	22.013	31.284	8.861	24.830	79.683	3.202
Molybdenum	2.757	0.894	1.514	0.541	0.690	3.154	0.184
Tin	0.037	0.002	0.091	0.023	0.008	0.025	0.008
Palladium	0.232	0.570	1.480	0.219	0.587	0.542	0.008
Antimony	0.034	0.022	0.029	0.023	0.013	nd	nd
Thallium	0.382	0.028	0.039	0.053	0.040	0.211	0.002
Tungsten	1.723	0.343	0.836	0.353	0.165	0.263	0.037

nd = not detectable

Table 2.3. Concentrations of metals determined in Garlic supplements by ICP-MS. The concentrations are represented in μg/day.

	Garlic			
Metals	Meijer	GNC	Nutrilite	Solaray
Lead	nd	0.031	0.021	0.140
Cadmium	0.137	0.030	0.012	0.068
Arsenic	0.107	0.127	0.001	0.058
Uranium	0.049	0.059	0.019	0.009
Chromium	0.678	0.283	0.085	0.504
Vanadium	0.126	0.081	0.025	0.196
Copper	9.318	1.433	2.920	6.407
Zinc	33.107	5.419	12.446	33.683
Molybdenum	1.606	0.243	0.326	0.490
Tin	0.005	0.005	0.016	0.012
Palladium	0.070	0.089	0.019	0.097
Antimony	0.005	0.025	0.002	nd
Thallium	0.010	0.004	0.003	0.009
Tungsten	0.256	0.056	0.047	nd

nd = not detectable

Table 2.4. Concentrations of metals determined in Ginkgo supplements by ICP-MS.

The concentrations are represented in μg/day.

	Ginkgo					
Metals	Nature's Way	Meijer	GNC	Nutrilite	Sundown	Solaray
Lead	12.545	0.269	0.127	0.078	7.367	0.019
Cadmium	2.886	0.042	0.030	0.020	0.041	0.011
Arsenic	3.080	0.127	0.560	0.175	0.813	0.146
Uranium	0.308	0.073	1.461	0.018	0.129	nd
Chromium	12.876	5.705	0.181	0.358	6.113	0.051
Vanadium	15.667	1.763	1.204	0.116	3.408	0.130
Copper	24.135	5.058	0.533	0.529	7.218	1.694
Zinc	98.493	11.137	140.999	3.461	11.558	10.117
Molybdenum	0.659	1.249	0.225	0.098	0.476	0.991
Tin	0.010	0.008	0.019	0.046	0.014	0.003
Palladium	1.262	0.279	0.468	0.064	0.422	nd
Antimony	0.061	0.050	0.017	0.006	0.052	nd
Thallium	0.315	0.012	0.013	0.031	0.088	nd
Tungsten	0.726	5.328	0.160	0.038	8.804	0.108

nd = not detectable

Table 2.5. Concentrations of metals determined in Ginseng supplements by ICP-MS. The concentrations are represented in μg/day.

	Ginseng						
Metals	Nature's Way	Meijer	GNC	Nutrilite	Sundown	Solaray	Natrol
Lead	0.128	9.226	1.213	0.379	0.135	1.686	0.439
Cadmium	0.121	0.177	0.076	0.025	0.021	0.158	0.020
Arsenic	0.696	0.598	0.363	0.128	0.217	0.193	0.059
Uranium	0.649	0.073	0.149	0.030	0.136	0.022	0.008
Chromium	5.641	3.723	4.102	0.732	2.483	0.897	1.010
Vanadium	3.774	2.429	0.606	0.178	0.895	0.650	0.148
Copper	7.342	10.986	3.873	2.722	4.032	4.289	0.012
Zinc	18.372	27.655	9.410	11.598	9.471	21.362	0.046
Molybdenum	1.401	1.066	0.652	0.786	0.370	0.450	0.087
Tin	0.050	0.035	0.110	0.034	0.077	0.012	0.017
Palladium	0.318	1.147	0.477	0.132	0.064	0.706	0.213
Antimony	0.111	0.039	0.063	0.009	0.014	0.021	0.010
Thallium	0.059	0.026	0.013	0.013	0.011	0.013	nd
Tungsten	0.473	1.052	1.800	0.261	0.259	0.839	1.593

nd = not detectable

Table 2.6. Concentrations of metals determined in Grape seed supplements by ICP-MS. The concentrations are represented in µg/day.

Grape Seed				
Metals	Nature's Way	GNC	Sundown	Solaray
Lead	0.055	0.202	0.819	0.084
Cadmium	0.020	0.051	0.017	0.007
Arsenic	0.071	0.514	0.349	0.046
Uranium	0.020	0.357	0.080	0.007
Chromium	0.114	1.412	1.631	0.425
Vanadium	4.398	0.647	1.299	1.830
Copper	6.515	1.816	12.181	3.741
Zinc	13.508	2.519	9.342	4.022
Molybdenum	0.691	0.705	0.199	0.010
Tin	0.031	0.087	0.111	0.097
Palladium	0.010	0.312	0.070	0.091
Antimony	0.318	0.122	0.029	0.133
Thallium	0.001	0.015	0.011	0.002
Tungsten	1.289	0.222	1.221	0.596

nd = not detectable

Table 2.7. Concentrations of metals determined in Kava kava supplements by ICP-MS.

The concentrations are represented in μg/day.

	Kava kava			
Metals	GNC	Sundown	Solaray	Natrol
Lead	0.576	2.346	0.331	0.245
Cadmium	0.131	0.273	0.006	0.016
Arsenic	0.113	0.341	0.034	0.091
Uranium	0.068	0.035	0.026	0.068
Chromium	5.409	3.292	0.603	0.692
Vanadium	0.670	2.318	0.173	0.300
Copper	7.402	13.371	1.067	1.487
Zinc	23.797	42.887	2.411	2.861
Molybdenum	0.545	0.188	0.030	0.132
Tin	0.102	0.030	0.014	0.003
Palladium	0.345	0.406	0.045	0.046
Antimony	0.007	0.009	nd	nd
Thallium	0.030	0.052	0.057	0.047
Tungsten	0.671	0.467	0.038	0.052

nd = not detectable

Table 2.8. Concentrations of metals determined in Saw Palmetto supplements by ICP-MS. The concentrations are represented in μg/day.

	Saw Palmetto					
Metals	Nature's Way	Meijer	GNC	Nutrilite	Sundown	Solaray
Lead	0.009	0.129	0.371	0.036	0.050	0.782
Cadmium	nd	nd	0.053	0.008	0.024	0.086
Arsenic	nd	nd	0.034	0.250	nd	0.139
Uranium	0.009	0.024	0.023	0.014	nd	0.009
Chromium	0.169	0.375	1.612	0.228	0.169	0.700
Vanadium	0.193	0.291	0.243	0.216	0.066	0.196
Copper	0.156	0.382	27.891	10.976	20.820	sat
Zinc	0.619	0.299	47.971	8.549	23.788	sat
Molybdenum	0.008	0.039	0.272	0.129	0.169	0.247
Tin	0.007	0.003	0.078	0.021	0.009	0.077
Palladium	nd	0.037	0.059	0.034	0.026	0.278
Antimony	nd	nd	0.022	nd	nd	nd
Thallium	nd	0.001	0.009	nd	nd	0.037
Tungsten	0.068	0.029	8.767	0.466	5.181	2.040

nd = not detectable

sat = saturated the detector

Table 2.9. Concentrations of metals determined in St. John's Wort supplements by ICP-MS. The concentrations are represented in μg/day.

	St. John's Wort						
Metals	Nature's Way	Meijer	GNC	Nutrilite	Sundown	Solaray	Natrol
Lead	1.175	0.206	5.831	0.068	0.588	0.351	0.146
Cadmium	0.054	0.080	2.115	0.047	0.092	1.114	0.156
Arsenic	0.131	0.080	0.320	0.078	0.565	0.145	0.828
Uranium	0.014	0.053	0.089	0.009	0.392	0.014	1.345
Chromium	0.769	0.969	4.725	0.219	6.047	0.340	3.926
Vanadium	0.373	0.354	3.487	0.063	2.935	0.248	3.513
Copper	20.090	14.903	34.648	19.788	14.150	9.534	22.462
Zinc	36.513	16.831	75.810	32.919	32.826	24.851	26.087
Molybdenum	0.318	0.355	1.123	0.279	0.693	5.443	3.035
Tin	0.031	0.018	0.132	0.017	0.305	0.013	0.637
Palladium	0.061	0.055	0.594	0.106	0.242	0.078	0.578
Antimony	0.014	0.025	0.044	0.003	0.013	0.005	0.060
Thallium	0.017	0.005	0.030	0.003	nd	0.010	0.033
Tungsten	0.107	0.524	36.006	0.094	0.314	8.933	0.476

nd = not detectable

Table 2.10. Bacteria and Fungi in the botanical supplements.

Manufacturer	Bacteria	Fungi
Nature's Way Echinacea	✓	✓
Nature's Way Korean ginseng	✓	-
Meijer garlic	✓	-
GNC garlic	✓	-
Solaray garlic	✓	-
Solaray St. John's wort	-	✓
Natrol echinacea	-	✓*
Natrol kava kava	✓	-
Sundown Korean ginseng	✓	-
Sundown echinacea	✓	-
Sundown garlic	✓	-

* Two different fungi

CHAPTER THREE

LIPID PEROXIDATION AND CYCLOOXYGENASE ENZYME INHIBITORY ACTIVITIES OF ACIDIC AQUEOUS EXTRACTS OF SOME DIETARY SUPPLEMENTS

Abstract

The botanical supplement market is growing at a fast pace with more and more people resorting to them for maintaining good health. Echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort are some of the popular supplements used for a variety of health benefits. These supplements are associated with various product claims, which suggest that they possess cyclooxygenase (COX) enzymes and lipid peroxidation inhibitory activities. COX enzymes are found to be at elevated levels in inflamed and cancerous cells. To test some of the product claims, we have analyzed selected supplements for their ability to inhibit COX-1 and -2 enzymes and lipid peroxidation in vitro. The supplements were extracted with acidified water (pH 2) at 37° C to simulate the gastric environment. The supplements tested demonstrated varying degrees of COX enzymes inhibitions (5-85% for COX-1 and 13-28% for COX-2). Interestingly, extracts of Garlic (Meijer), Ginkgo (Solaray), Ginseng (Nature's Way, GNC, Nutrilite, Solaray, Natrol), Kava kava (GNC, Sundown, Solaray) and St. John's wort (Nutrilite) selectively inhibited COX-2 enzyme. These supplements also inhibited lipid peroxidation in vitro (5-99%). Our results indicated that the consumption of these botanical supplements studied possess health benefits.

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Introduction

The sale of the botanical supplements has increased significantly over the past years (Eisenberg et al., 1998). People have resorted to botanical supplements to maintain good health and vitality. Many botanical supplements claim to perpetuate good health but very little scientific research has been accomplished to corroborate these statements. Research in regard to the efficacy of these supplements would add further notoriety. Echinacea, garlic, ginkgo, ginseng, grape seed extract, kava kava, saw palmetto and St. John's wort supplements are among the popular supplements sold in the US (Blumenthal, 1999). These supplements state to promote immune function, cardiovascular health, mental alertness, endurance, wellbeing, prostate health, enhance the mood and provide anti-oxidant protection (Table 3.1). Also, it suggests that these botanicals might possess anti-oxidant and anti-inflammatory properties, which aid in general good health.

Cyclooxygenase (COX) enzymes play an important role in the inflammatory processes. There are two isoforms of the COX enzyme, COX-1 and COX-2. COX-1 is the constitutive form of the enzyme and is responsible for basic regulatory functions in cells and is involved in the production of prostaglandins (Cryer and Dubois, 1998). Prostaglandins are also responsible for the production of gastric secretions. COX-2 is the inducible form, which is produced in response to inflammation (Lipisky, 1999). Therefore, selective inhibition of COX-2 enzyme is desirable to prevent the undesirable side effects of COX-1 inhibition such as gastric ulcerations. The antioxidant property can be attributed either to inhibition of the production of reactive oxygen species or to scavenging of the free radicals (Arora et al., 1998). Lipid peroxidation is one of the major causes of free radical generation in vivo. Oxidative stress has been implicated in

many of the chronic diseases. Therefore, anti-oxidant activity of these botanicals can play a major role in imparting good health.

It was reported that these botanicals possessed either antioxidant property or inhibition of COX enzymes activity or both. For example, echinacea was demonstrated to possess antioxidant property (Hu and Kitts, 2000; Facino et al., 1995) and the polyalkamides from it were shown to inhibit microsomal COX enzyme in vitro (Muller-Jakic et al., 1994). Similarly, several garlic compounds have been reported to effectively suppress LDL oxidation in vitro (Lau, 2001). *Ginkgo biloba* extract (EGb 761) was demonstrated to possess antioxidant property due to the inhibiton of free radical formation as well as by scavenging of the free radicals (Pietri et al., 1997; Shen et al., 1996). It was reported that a standardized extract of ginseng reduced lipid peroxidation (Cabral de Oliveira et al., 2001). The proanthocyanidins from grape seed extract have been reported to inhibit lipid peroxidation and to modulate the activity of enzyme systems including COX and lipooxygenase enzymes (Bors and Saran, 1987; Kolodziej et al., 1995). They were found to be potent free radical scavengers (Ricardo da Silva et al., 1991; Bagchi et al., 1998; Yamaguchi et al., 1999). Saw palmetto berry extract (SPBE) was observed to inhibit COX-2 expression, which is associated with an increased incidence of prostate cancer (Goldman et al., 2001). Based on the wide spread health attributes associated with these botanicals, we have investigated their ability to inhibit COX enzymes and lipid peroxidation in vitro.

In our study, the botanical supplements were extracted separately with acidified water to simulate the gastric environment (pH = 2, 37° C). The gastric environment is acidic in nature when food is not ingested. Fasting gastric pH has been well studied

(Malagelada et al., 1976; Malagelada et al., 1977) and the generally accepted value for fasting gastric pH is approximately 2 (Dressman et al., 1990).

Materials and methods

Botanical supplement samples

Echinacea, garlic, *Ginkgo biloba*, ginseng, grape seed extract, kava kava, Saw palmetto and St. John's wort, manufactured by Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray and Natrol, were purchased in 2002 and 2003 from stores in Michigan, Illinois and Indiana (Table 3.1).

Preparation of extracts for in vitro assays

The supplements tested were in the form of capsules, tablets or soft gels. For capsules and soft gels, the shells were removed before extraction. The tablets were powdered and used for extraction. Three unit (1 unit = 1 tablet/capsule/soft gel) contents of each supplement were weighed and extracted with 25 mL of acidified water (pH = 2) by placing it on a shaker for 6 h at 37° C, and centrifuged. The resulting extracts were lyophilized and the dry extracts were used to perform in vitro bioassays (Table 3.2). The extraction was carried out at pH = 2 and 37° C to simulate the gastric environment.

Cyclooxygenase enzyme inhibitory assay

COX-1 activity was assessed using an enzyme preparation from ram seminal vesicles (Oxford Biomedical Research, Inc., Oxford, MI). COX-2 activity was determined using a preparation of human prostaglandin H synthase isozyme 2 (hPGHS-2) cloned in insect cells. COX assays were carried out by monitoring the rate of oxygen uptake in an micro chamber and the oxygen electrode (Instech Laboratories, Plymouth Meeting, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs

Instrument, Inc., Yellow Springs, OH) as reported earlier (Wang et al., 2000; Seeram et al., 2001; Francis et al., 2004). Each assay mixture contained 0.6 mL 0.1M Tris buffer (pH 7), 1 mM phenol, 17 μg hemoglobin. The test samples (6 μL) and the enzyme (10 μL for COX-1 and 30 μL for COX-2) were incubated for 3 min and then with 10 μL of arachidonic acid solution (0.25 mg/0.25 mL Tris buffer) to initiate the reaction. Data were recorded using Quicklog for Windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA). The samples were tested at 25 and 100 μg/mL. Rofecoxib (Vioxx®) (1 μg/mL), Celecoxib (Celebrex®) (1 μg/mL), Naproxen (1.5 μg/mL) and Aspirin (108 μg/mL) were assayed as positive controls.

Lipid peroxidation inhibitory assay

The lipid peroxidation assay was conducted by using a model liposome and its oxidation using flurorescence spectroscopy. Synthetic 1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine (SLPC) (Avanti Polar Lipids, Alabaster, AL) was the lipid substrate used. The lipid and the fluorescent probe, 3-[p-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid (DPH-PA) (Molecular Probes, Inc., Eugene, OR), were dissolved in DMF and dried under vacuum at room temperature. The resulting lipid film was hydrated with a buffer (500 μL containing 0.15M NaCl, 0.01M MOPS (pH 7.0) and 0.1 mM EDTA). Large Unilamellar Vesicles (LUVs) were prepared by subjecting the resuspended mixture to 10 freeze-thaw cycles using a dry ice/ethanol bath, followed by extrusion (29 times) through a 100-nm pore size membrane in a Lipofast extruder apparatus (Avestin Inc., Ottawa, Canada) (Arora et al., 1997). The fluorescent intensity assay described by Arora and Strasburg (1997) was used to assess the antioxidant efficacy of the samples. In the assay, the peroxidative degradation of the probe DPH-PA

is indicated by the decrease in fluorescence and is used to monitor the sensitivity of the membrane towards oxidative stress. The final assay volume was 2 mL, consisting of 100 μL HEPES buffer (50 mM HEPES and 50 mM TRIS), 200 μL 1M NaCl, 1.645 mL N_2 sparged water, 20 μL of test sample or DMSO (blank) and 15 μL aliquot of liposome suspension. Peroxidation was initiated by the addition of 20 μL $FeCl_2$. 4 H_2O (0.5 mM). Positive controls used were BHA, BHT and TBHQ at 1.80 μg/mL, 2.20 μg/mL and 1.66 μg/mL respectively and test samples at 25 or 10 μg/mL. Fluorescence was measured at 384 nm and monitored at 0, 1, 3 and every 3 min thereafter up to 21 min using a Turner Model 450 Digital Fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity over time indicated the rate of peroxidation. Relative fluorescence (F_t/F_0) was calculated by dividing the fluorescence value at a given point (F_t.) by that at t = 0 min (F_0).

Results

The supplements were extracted separately with acidified water (pH=2) for 6 h at 37° C, centrifuged and the resulting extracts were lyophilized. The weight of lyophilized extracts varied among supplements (Table 3.2). The amount of extracts used in the in vitro assay was calculated based on the recommended dose of the specific supplement per day. Therefore, the standardized amount of extract per kg body weight varied between 6 and 49 mg, depending on the supplement. For convenience and ease of conducting the bioassays, the concentration selected for COX and lipid peroxidation bioassays was 25 μg/mL.

Garlic (Meijer, Sundown), ginkgo (Solaray), ginseng (Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray, Natrol), kava kava (GNC, Sundown, Solaray, Natrol)

and St. John's wort (Nutrilite) showed only marginal COX-2 enzyme inhibitory activity at 25 μg/mL. Therefore, the assays were repeated at 100 μg/mL for these extracts. Since, these extracts had COX-2 enzyme inhibitory activity at 100 μg/mL, the COX-1 enzyme inhibitory assay was conducted only at 100 μg/mL. At this concentration, most extracts displayed 5-85% of COX-1 and 13-28% of COX-2 enzyme inhibitory activities. The results of COX enzymes inhibitory activities of the standards and extracts are presented in figures 3.1a - 3.1c. Garlic (Meijer, Sundown), ginkgo (Solaray), ginseng (Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray, Natrol), kava kava (GNC, Sundown, Solaray, Natrol) and St. John's wort (Nutrilite) exhibited selective COX-2 enzyme inhibition at 100 μg/mL (Figure 3.1b). However, extracts of garlic (Sundown), ginseng (Nature's Way, Meijer, Sundown), kava kava (Natrol) and St. John's wort (Nutrilite) demonstrated COX-1 enzyme inhibition (Figure 3.1b). The ginseng (Meijer) extract gave a higher COX-1 (43%) enzyme inhibition than COX-2 (28%). Echinacea (Meijer), garlic (GNC, Nutrilite), ginkgo (Meijer, GNC), grape seed (GNC, Nature's Way, Solaray), saw palmetto (GNC, Sundown, Nutrilite) and St. John's wort (Natrol, Nature's Way) extracts exhibited selective COX-1 enzyme inhibition (Figure 3.1c).

The inhibition of lipid peroxidation was tested at 25 μg/mL for all supplement extracts (figures 3.2a – 3.2f). Most of the extracts tested at 25 μg/mL inhibited lipid peroxidation, which included extracts from echinacea (27-99%), garlic (14-32%), ginkgo (57-96%), ginseng (11-91%), grape seed (68-120%), kava kava (13-52%), saw palmetto (5-87%) and St. John's wort (80-93%) supplements. The extracts, which demonstrated a higher lipid peroxidation inhibitory activity than the standards at 25 μg/mL were assayed again at 10 μg/mL (figure 3.2g).

Discussion

Selective inhibition of COX-2 enzyme is desirable for a supplement. This is because COX-2 enzyme is normally induced in response to inflammation. It is also found at elevated levels in many human cancers, especially colorectal cancers (Hsi et al., 1999). Therefore, COX-2 enzyme inhibitors are not only ideal antiinflammatory agents but also useful in the prevention and progression of several types of cancers. The COX-1 enzyme is expressed constitutively in many tissues (Smith and DeWitt, 1996) and it is also involved in the production of prostaglandins. Inhibition of COX-1 enzyme also implicated in the prevention of cancer (Hsi et al., 1999). But the only adverse effect of COX-1 inhibitors is the gastric ulcerations, as prostaglandins are also involved in the production of protective mucus in the stomach.

There is a wealth of evidence that Non Steroidal Anti-Inflammatory Drugs (NSAIDs) can prevent colorectal cancers (Luk, 1996; Kate et al., 2002; Herendeen and Lindley, 2003). Most of the NSAIDs inhibit both isoforms of COX and thus, gastric ulcer is usually associated with its use. But it has been suggested that inhibition of both isoforms of COX may have important protective effects against colorectal cancer (Watson, 1998; Slattery et al., 2004).

The lipid peroxidation has been implicated in many of the chronic illnesses. Prevention of free radical generation or its scavenging can be beneficial in maintaining a good health. Inhibition of lipid peroxidation in vivo can prevent the free radicals involved in oxidative tissue damage. Inhibition of COX enzymes and lipid peroxidation by the extracts produced from the supplements studied indicate that could play a role in maintaining good health. For example, the extracts of garlic (Meijer), ginkgo (Solaray),

ginseng (Nature's Way, GNC, Nutrilite, Solaray, Natrol), kava kava (GNC, Sundown, Solaray) and St. John's wort (Nutrilite) selectively inhibited the COX-2 enzyme and lipid peroxidation at the recommended dose per day. Hence, these supplements may be beneficial in the prevention and or treatment of inflammation and cancer. Further research should be directed to the identification of the active ingredients in the acidic aqueous extracts of these supplements and their effective dosage.

Table 3.1. Health claims reported on the bottle of each supplement studied.

Botanical	Manufacturer	Claims
Echinacea	Nature's Way	Supports the Immune system
	Meijer	Stimulates the Immune system
	GNC	-
	Nutrilite	Supports body's natural resistance
	Sundown	Healthy Immune function
	Solaray	-
	Natrol	Supporting the body's defense system
Garlic	Meijer	Helps retain normal, healthy cholesterol levels
	GNC	Garlic provides dietary support for normal healthy cardiovascular function
	Nutrilite	Supports overall cardiovascular health
	Sundown	Healthy heart function
	Solaray	-
Ginkgo	Nature's Way	For mental function
	Meijer	Cerebral circulation
	GNC	*Ginkgo biloba* supports increased blood flow to the brain
	Nutrilite	Has been studied to improve blood flow to the brain
	Sundown	Mental alertness
	Solaray	Intended to provide dietary support to help promote brain circulation for enhanced neuro activity
Ginseng	Nature's Way	Enhances physical endurance and mental vitality
	Meijer	Physical and mental stress
	GNC	-
	Nutrilite	Siberian ginseng has been studied for its effect on work endurance
	Sundown	Energy and endurance
	Solaray	-
	Natrol	Siberian ginseng enhances physical and mental resistance to environmental stress while fortifying general endurance

Table 3.1 (contd.). Health claims reported on the bottle of each supplement studied.

Botanical	Manufacturer	Claims
Grape seed	Nature's Way	Powerful anti-oxidant
	GNC	-
	Sundown	Superior anti-oxidant protection
	Solaray	-
Kava kava	GNC	-
	Sundown	Calm well-being
	Solaray	-
	Natrol	Calming benefits of kava kava after a stressful day. Its affects relaxation without hampering memory and reaction time.
Saw palmetto	Nature's way	Prostate Health
	Meijer	Prostate Health
	GNC	-
	Nutrilite	For men, Saw palmetto and Pumpkin seed oil support normal prostate function. Nettle root supports normal urinary flow
	Sundown	Prostate and urinary health
	Solaray	-
St. John's wort	Nature's Way	Positive mood
	Meijer	Mood enhancer
	GNC	-
	Nutrilite	St. John's wort is clinically proven natural approach that helps support a healthy emotional balance and well being
	Sundown	Mood enhancement
	Solaray	-
	Natrol	St. John's wort plays a role in mood enhancement and maintaining a healthy positive mental outlook

Table 3.2. The yield of extract obtained from each supplement after extraction with acidic water at pH = 2 and 37° C.

Botanical	Manufacturer	**Weight of extract/unit (mg)**
Echinacea	Nature's Way	154.70
	Meijer	110.10
	GNC (*Echinacea purpurea*)	64.70
	Nutrilite (Triple Guard Echinacea)	36.77
	Sundown	90.93
	Solaray (Echinacea root)	32.60
	Natrol	27.07
Garlic	Meijer	180.73
	GNC (Odorless Garlic)	102.90
	Nutrilite	46.10
	Solaray	32.60
	Sundown	85.23
Ginkgo	Nature's Way	64.80
	Meijer	192.60
	GNC	37.77
	Nutrilite (*Ginkgo biloba* and Dha)	27.30
	Sundown	56.93
	Solaray	15.77
Ginseng	Nature's Way (Korean ginseng)	146.67
	Meijer (Siberian ginseng)	13.37
	GNC (Siberian ginseng)	27.00
	Nutrilite (Siberian ginseng with *Ginkgo biloba*)	342.43
		144.03
	Sundown (Korean ginseng)	4.83
	Solaray (Siberian ginseng)	40.37
	Natrol (Siberian ginseng)	

Table 3.2 (contd.). The yield of extract obtained from each supplement after extraction with acidic water at pH = 2 and 37° C.

Botanical	Manufacturer	Weight of extract/unit (mg)
Grape seed	Nature's Way	93.10
	GNC	39.17
	Sundown	221.20
	Solaray	49.77
Kava kava	GNC	65.97
	Sundown	80.67
	Solaray	51.33
	Natrol	29.33
Saw palmetto	Nature's Way	2.43
	Meijer	232.20
	GNC	104.43
	Nutrilite (Saw palmetto and Nettle root)	43.17
	Sundown	36.60
	Solaray	101.40
St. John's wort	Nature's Way	228.97
	Meijer	184.87
	GNC	205.20
	Nutrilite (St. John's wort with Lemon balm)	311.10
	Sundown	213.77
	Solaray	23.97
	Natrol	137.47

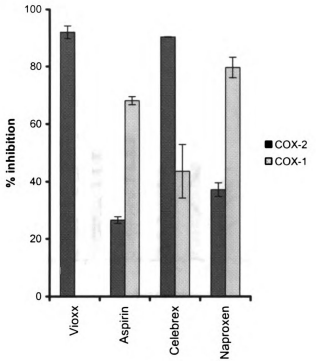


Figure 3.1a. Inhibition of COX enzymes by Vioxx (1μg/mL), Aspirin (108μg/mL), Celebrex (1 μg/mL), Naproxen (1.5 μg/mL). Vertical bars represent the standard deviation of each data point (n=2).

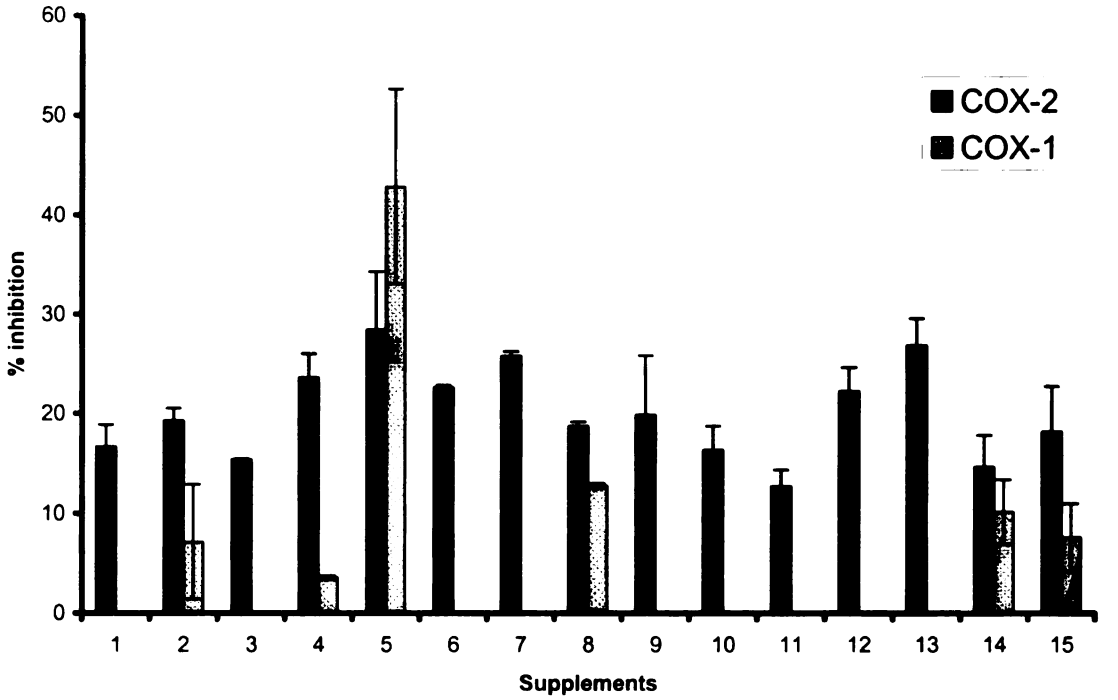


Figure 3.1b. Inhibition of COX-1 and 2 enzymes by the acidic aqueous extract prepared from botanical supplements studied at 100 μg/mL. The extracts are 1 (Meijer garlic), 2 (Sundown garlic), 3 (Solaray ginkgo), 4 (Nature's Way ginseng), 5 (Meijer ginseng), 6 (GNC ginseng), 7 (Nutrilite ginseng), 8 (Sundown ginseng), 9 (Solaray ginseng), 10 (Natrol ginseng), 11 (GNC kava kava), 12 (Sundown kava kava), 13 (Solaray kava kava), 14 (Natrol kava kava) and 15 (Nutrilite St. John's wort). Vertical bars represent the standard deviation of each data point (n=2).

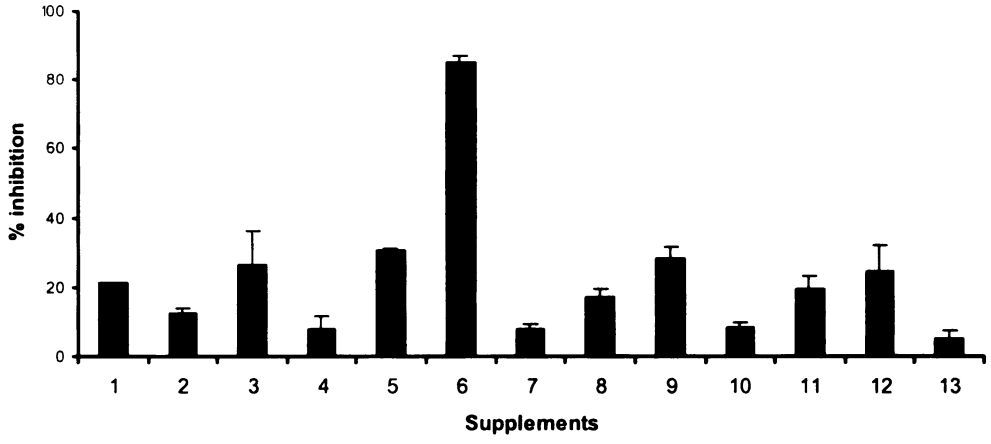


Figure 3.1c. Inhibition of COX – 1 enzyme by the acidic aqueous extract prepared from botanical supplements studies at 100 μg/mL. The extracts are 1 (Meijer echinacea), 2 (GNC garlic), 3 (Nutrilite garlic), 4 (Meijer *Ginkgo biloba*), 5 (GNC *Ginkgo biloba*), 6 (GNC grape seed), 7 (Nature's Way grape seed), 8 (Solaray grapenol), 9 (GNC Saw palmetto), 10 (Sundown Saw palmetto), 11 (Nutrilite Saw palmetto), 12 (Natrol St. John's wort) and 13 (Nature's Way St. John's wort). Vertical bars represent the standard deviation of each data point (n=2).

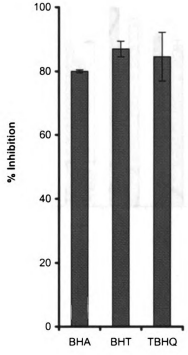


Figure 3.2a. Inhibition of lipid peroxidation at t = 21 min. Standards (BHA, BHT and TBHQ at 1.80 μg/mL, 2.20 μg/mL and 1.66 μg/mL respectively). Vertical bars represent the standard deviation of each data point (n=2).

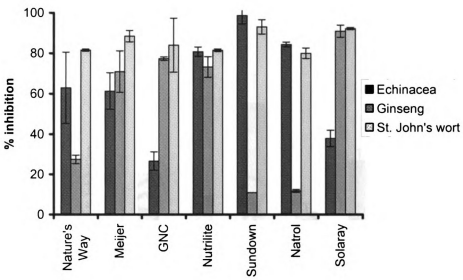


Figure 3.2b. Echinacea, Ginseng and St. John's wort supplements at 25 µg/mL. Vertical bars represent the standard deviation of each data point (n=2).

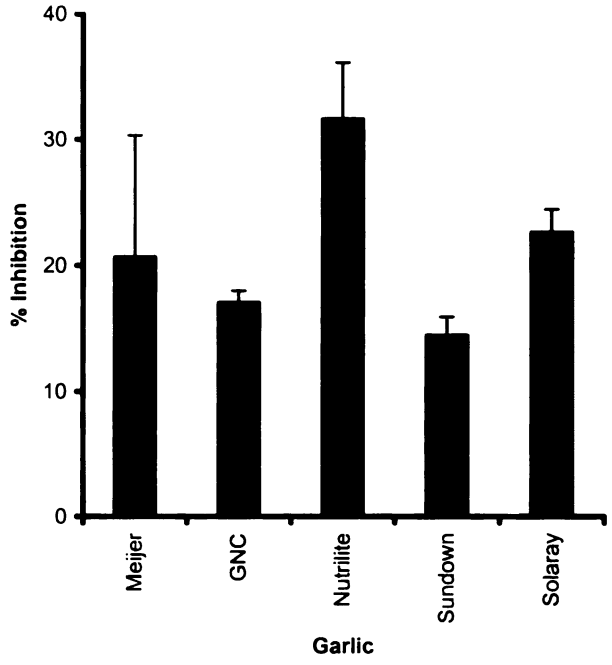


Figure 3.2c. Garlic supplements at 25 μg/mL. Vertical bars represent the standard deviation of each data point (n=2).

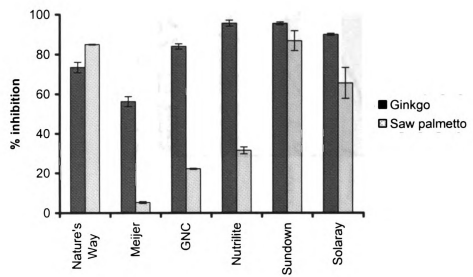


Figure 3.2d. Gingko and Saw palmetto supplements at 25 μg/mL. Vertical bars represent the standard deviation of each data point (n=2).

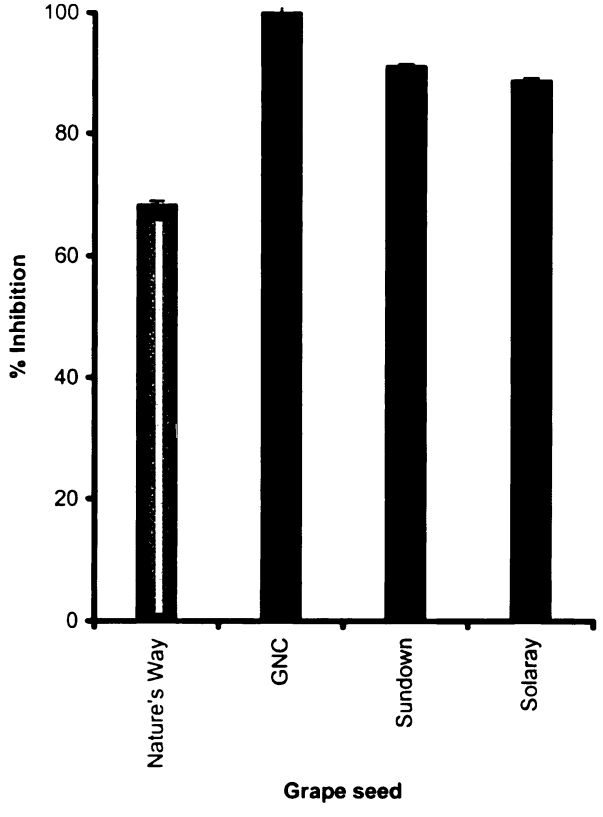


Figure 3.2e. Grape seed supplements at 25 μg/mL. Vertical bars represent the standard deviation of each data point (n=2).

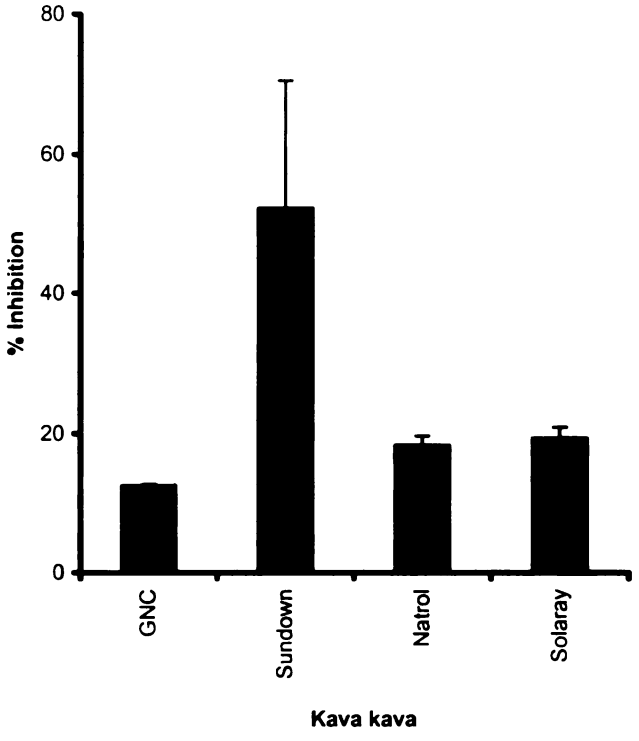


Figure 3.2f. Kava kava supplements at 25 µg/mL. Vertical bars represent the standard deviation of each data point (n=2).

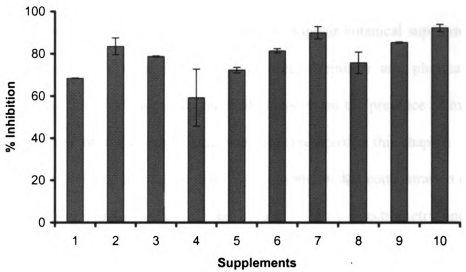


Figure 3.2g. The active extracts at 10 µg/mL. The samples are 1 (Solaray *Ginkgo biloba*), 2 (Nutrilite *Ginkgo biloba* and Dha), 3 (Sundown *Ginkgo biloba*), 4 (Solaray Siberian ginseng), 5 (Sundown Grape seed), 6 (GNC Grape seed), 7 (Solaray Grapenol), 8 (Sundown St. John's wort), 9 (Solaray St. John's wort) and 10 (Meijer St. John's wort). Vertical bars represent the standard deviation of each data point (n=2).

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

Botanical supplements are popular and are used to treat or prevent illnesses and improve general health. Among them, echinacea, garlic, ginkgo, ginseng, grape seed, kava kava, saw palmetto and St. John's wort are the popular botanical supplements sold in the US. In **Chapter One**, the traditional uses, chemistry and pharmacological properties of these botanicals were summarized. Reports on the presence of metals and microorganisms present in the supplements were also presented in this chapter. Based on this review, it was decided to analyze these supplements for the concentration of metals present in them using Inductively Coupled Plasma – Mass Spectrometry and for the presence of bacteria and fungi. These were also analyzed for cyclooxygenase enzyme and lipid peroxidation inhibitory activities, based on their reported pharmacological activities. For this purpose, echinacea, garlic, ginkgo, ginseng, grape seed, kava kava, saw palmetto and St. John's wort supplements manufactured by Nature's Way, Meijer, GNC, Nutrilite, Sundown, Solaray and Natrol were purchased and subjected to analysis.

The supplements were subjected to sample digestion for metal analysis and were analyzed for metals using Inductively Coupled – Plasma Mass Spectrometry. The data from the ICP-MS analyses were presented in **Chapter Two**. The results indicated that the supplements studied did not contain unacceptable concentrations of lead, cadmium, arsenic, uranium, chromium, vanadium, copper, zinc, molybdenum, palladium, tin, antimony, thallium and tungsten. Analysis of these supplements for the presence of microorganisms indicated that bacteria were present in Nature's Way Korean ginseng,

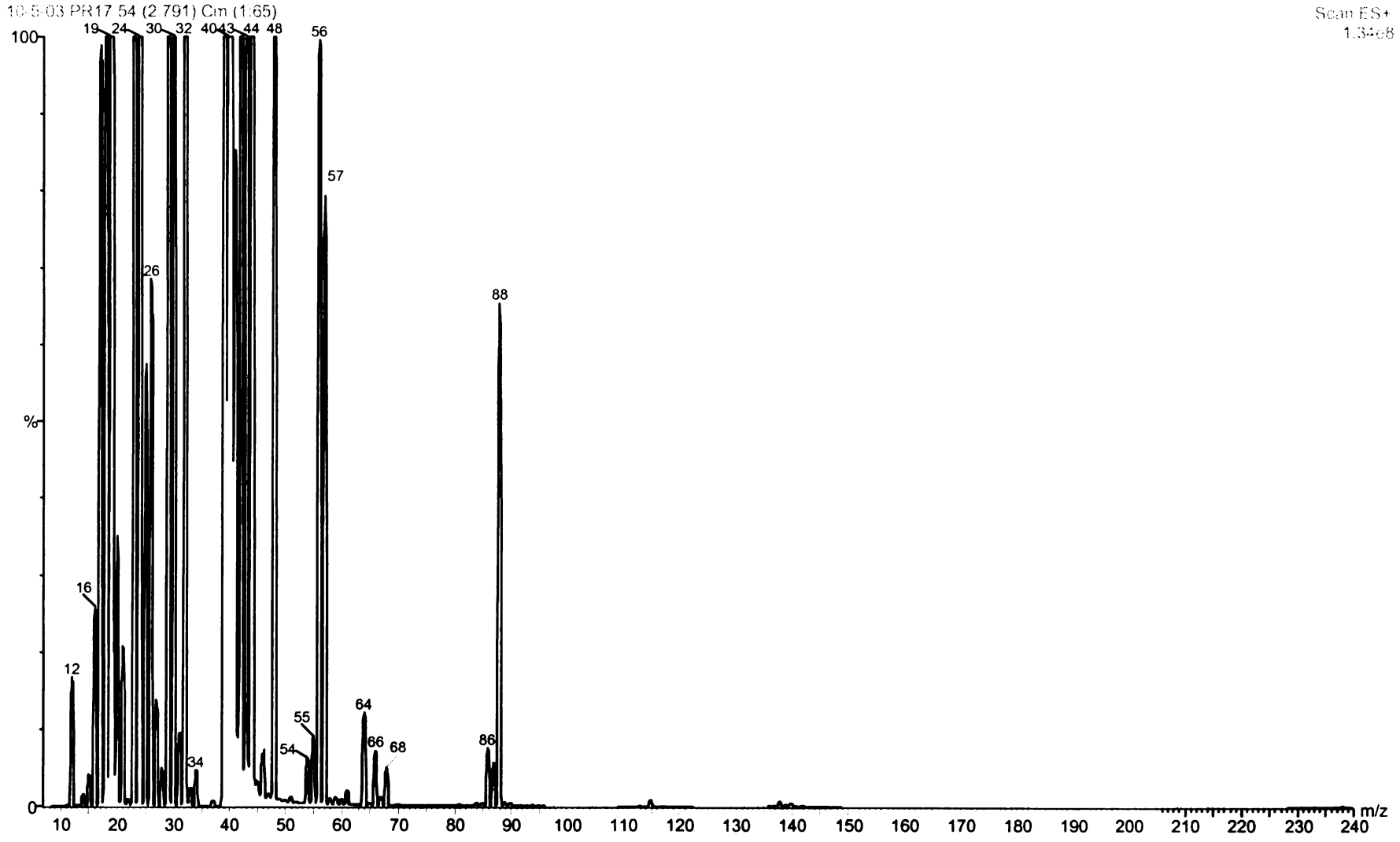
Meijer garlic, GNC garlic, Solaray garlic, Natrol kava kava, Sundown Korean ginseng, Sundown echinacea and Sundown garlic. Fungi were present in Nature's Way echinacea, Solaray St. John's wort, Natrol echinacea and Sundown saw palmetto.

The product label of these botanical supplements suggested that they possess cyclooxygenase enzymes and lipid peroxidation inhibitory properties. The reports of the pharmacological activities of these botanicals also suggested the same. Therefore, in vitro assays were carried out on extracts of the supplements to corroborate such claims. To accomplish this, the supplements were extracted with acidified water (pH 2) at 37° C to simulate the gastric environment. The results of these bioassays were presented in **Chapter Three**. The supplements tested demonstrated varying degrees of COX enzymes inhibitions (5-85% for COX-1 and 13-28% for COX-2). It was interesting to note that extracts of Garlic (Meijer), Ginkgo (Solaray), Ginseng (Nature's Way, GNC, Nutrilite, Solaray, Natrol), Kava kava (GNC, Sundown, Solaray) and St. John's wort (Nutrilite) specifically inhibited COX-2 enzyme. These supplements also inhibited lipid peroxidation in vitro (5-99%).

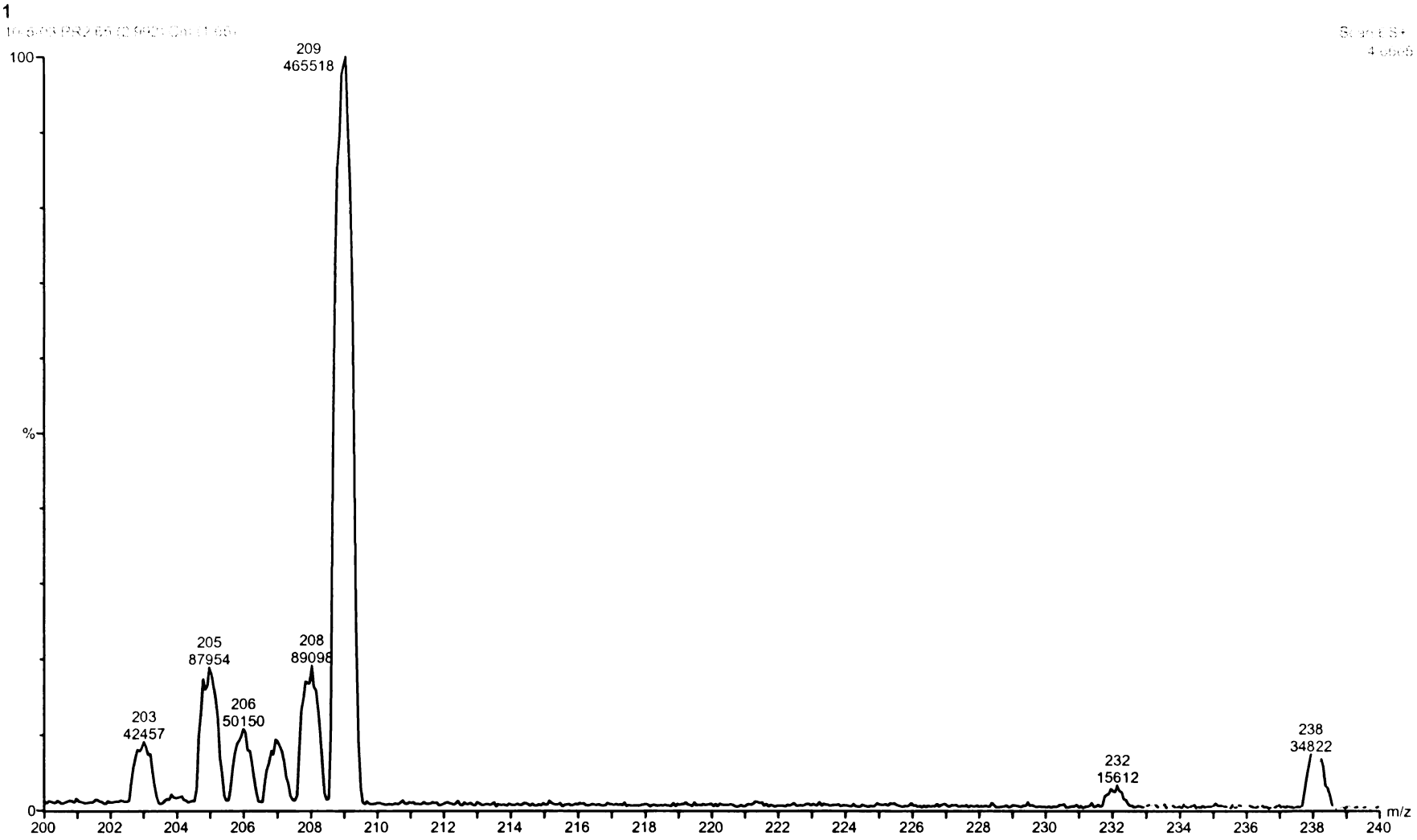
Research on the food safety aspects of the botanical supplements has revealed that these supplements are safe to consume with regard to metal contamination, though further research is required in regard to microbial contamination, i.e., to type the microorganisms present in them. Research on the health benefits on these supplements has revealed that these supplements possess cyclooxygenase enzymes and lipid peroxidation inhibitory properties, which is helpful in the prevention or treatment of inflammation and also certain types of cancer. Further work is essential to determine the

identity, concentrations and effective dose of the active ingredients present in the acidified aqueous extracts of these botanicals.

Appendix



Appendix 1. An example of full mass scan and the range of m/z ratio of 200 to 240 expanded.



Appendix 1 (contd.). An example of full mass scan and the range of m/z ratio of 200 to 240 expanded.

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