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ISOLATION AND CHARACTERIZATION OF ANTI-OXIDANT AND ANTI-INFLAMMATORY CONSTITUENTS FROM ECHINACEA PURPUREA (L.) MOENCH

presented by

Laura Jean Clifford

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Major professor

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ISOLATION AND CHARACTERIZATION OF ANTI-OXIDANT AND ANTI-INFLAMMATORY CONSTITUENTS FROM *ECHINACEA PURPUREA* (L.) MOENCH

By

Laura Jean Clifford

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF ANTI-OXIDANT AND ANTI-INFLAMMATORY CONSTITUENTS FROM *ECHINACEA PURPUREA* (L.) MOENCH

By

Laura Jean Clifford

A variety of anecdotal claims have been attributed to *Echinacea purpurea* (L.) Moench, including immune systems enhancement and pain and fever relieving properties. However, questions still remain as to the identity and mode of action of the compounds responsible for these biological effects. Research to identify specific constituents responsible for reported anecdotal claims has established several compounds with significant biological potential. Alkamides (undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide, dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide) were isolated from *E. purpurea* roots and examined in in vitro model systems. Significant cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activities, ranging from of 36-60% and 15-46%, respectively, were observed for these compounds at a concentration of 100 μg/mL. The alkamides from *E. purpurea* were mosquitocidal against *Aedes aegyptii* L. larvae at 10 and 100 μg/mL. Flavonoids (quercetin-3-*O*-glucoside, quercetin-3-*O*-

rutinoside, and kaempferol-3-*O*-robinobioside) and anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside), were isolated from methanolic extracts of lyophilized *E. purpurea* flowers and examined for antioxidant and COX-1 and COX-2 inhibitory activities. Four additional reported constituents of *E. purpurea*, caffeic acid and the caffeoyl derivatives caftaric acid, chlorogenic acid, and cichoric acid were also tested. The anthocyanins demonstrated the greatest cyclooxygenase inhibitory activities at 100 μg/mL, with inhibitions of COX-1 and COX-2 ranging from 23-28%. Co-treatment of RAW 264.7 macrophages with several of the isolated compounds (at 1, 10, 50, and 100 μg/mL) and 100 or 1000 ng/mL lipopolysaccharide (LPS) suppressed induction of the pro-inflammatory cytokines IL-6 and TNF-α. Cytotoxicity was noted at 100 μg/mL for all four alkamides, based upon results obtained from a concurrent MTT assay. Cells treated with compounds from *E. purpurea* did not induce IL-6 or TNF-α production; however, co-treatment of macrophages with caffeoyl derivatives (50 and 100 μg/mL) and LPS (100 and 1000 ng/mL) resulted in an increase in IL-6 and TNF-α levels, suggesting potential immune-enhancing activity. The alkamides demonstrated the greatest ability to reduce LPS-induced IL-6 and TNF-α production. The anti-inflammatory activity of the alkamides reported here supports the claims of pain and fever relief associated with *Echinacea* use. The opposing activities noted for compounds from *E. purpurea* demonstrate the importance of testing individual compounds rather than crude extracts.

To all individuals who see the invisible and do the impossible.

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INTRODUCTION

Establishing the safety and efficacy of natural product supplements through scientific validation is of primary concern to both consumers and manufacturers of phytoceuticals. Although one-half of the world's populace uses traditional medicines as a primary source of medical treatment (Wildman, 2001), Americans have only recently begun to add significant quantities of natural product supplements to their normal medical regimens (Borchers et al., 2000; Miller, 1998). The increase in the popularity of nutraceuticals and phytoceuticals as an addition or alternative to Western medicine might be attributed to the public's heightened awareness of preventable diseases, such as diabetes, cancer, and heart disease, as well as to the increase in the percentage of older Americans (Wildman, 2001). Alternative medicines have the potential to improve quality of life and provide a new means of disease prevention or management (Ness et al., 1999); however, phytoceuticals might also interfere with mainstream medical treatments.

Nutraceuticals do not require testing for safety and efficacy prior to marketing (Percival, 2000; Zink and Chaffin, 1998). As a result, the active components in many commercially available products have not been identified (Mazza and Cottrell, 1999; Melchart et al., 1995). Due to the lack of standardization in the phytoceutical industry (Perry et al., 2001) and the unstable nature of compounds in natural product extracts (Kim et al., 2000a; Nüsslein et al., 2000), greater regulation is needed to protect consumers. While it has been suggested that *Echinacea* extracts and products should be standardized in order

to provide a consistent product to consumers, many natural product supplements do not undergo rigorous quality or standardization testing (Bergeron et al., 2000; Bauer et al., 1998).

Regulatory issues have become increasingly important as a result of the soaring increase in the phytoceutical market, the lack of standardized extracts, the potential instability of bioactive compounds in extracts, and the lack of safety and/or efficacy testing. In 1993, the Food and Drug Administration (FDA) implemented the Nutrition Labeling and Education Act (NLEA), which distinguished between the definitions of "food" and "drug" (Childs, 2001) and provided the basis for stronger regulatory control by the FDA. In response to the NLEA, the dietary supplement industry pursued the establishment of a separate category for dietary supplements with regard to labeling and advertising claims. The result was the formation of the Dietary Supplement Health and Education Act (DSHEA) (Wildman, 2001; Stephen, 1998). The recently proposed Nutraceutical Research and Education Act defines nutraceuticals as "a dietary supplement, food, or medical food that possesses health benefits and is safe for human consumption in such quantity and with such frequency as required to realize such properties" (Henry, 1999).

Heightened regulatory control and the establishment of a formal definition of nutraceuticals have provided a strong incentive for scientists and the natural product supplement industry to establish scientific credibility for herbal supplement use. Nutraceuticals have been perceived to be inherently safe by consumers because they are "natural," even without adequate toxicity testing

(Zink and Chaffin, 1998). The impetus to prove the safety and efficacy of nutraceuticals will provide solid scientific data to substantiate and/or disprove anecdotal claims of biological effects. Identification of specific, active components in natural product supplements is a vital area of research, due in part to the potential for drug-herb interactions (Miller, 1998).

In a recent survey, an estimated four in ten Americans used some type of unconventional treatment, spending approximately $34 billion on alternative medicines (Ness et al., 1999). *Echinacea* supplements and mixtures of herbal supplements containing *Echinacea* are among the most popular natural product supplements in the United States (Barrett et al., 1999). Annual sales of *Echinacea* nutraceuticals are estimated at $300 million (Gunning, 1999) and are expected to increase in forthcoming years, allowing *Echinacea* preparations to remain a top-selling nutraceutical (Stephen, 1998).

Echinacea purpurea (L.) Moench, *Echinacea angustifolia* DC, and *Echinacea pallida* Nutt. are used to the greatest extent in phytoceutical products (Barrett et al., 1999; Bauer and Wagner, 1991). *Echinacea* preparations are sold in the form of tablets, capsules, tinctures (alcoholic extracts), juices, and teas (Gunning, 1999; Li and Wang, 1998). Alcoholic extracts of *Echinacea* roots and above-ground portions of the plant are most commonly consumed. In Germany, more than 800 *Echinacea*-based extracts are commercially available (Bauer, 1998; Lienert et al., 1998), and the number of preparations available in the United States is increasing. Many nutraceuticals have come under scrutiny due to the lack of credible pharmacological and chemical data supporting their use. A

limited number of studies have been conducted to substantiate anecdotal claims associated with *Echinacea* use (Borchers et al., 2000). The claims of pain relief and general immune stimulation have been evaluated to some extent (Bauer, 1998; Bauer and Wagner, 1991; Proksch and Wagner, 1987). Anti-viral, antioxidant, and anti-inflammatory activities have been reported for various *Echinacea* extracts (Facino et al., 1995; Bauer & Wagner, 1991; Wagner et al., 1988; Luettig et al., 1989). Despite these efforts, additional research is needed in order to validate the anecdotal health claims attributed to *Echinacea* dietary supplements.

Anecdotal claims of immune enhancement, fever relief, and alleviation of pain have been associated with the use of *E. purpurea* preparations. The bioactive compounds responsible for anecdotal claims have not been fully eluciated to date. This study was undertaken in order to identify bioactive constituents of *E. purpurea* through the isolation, structure elucidation, and biological testing of the active compounds in *E. purpurea* roots and flowers. Five primary objectives were defined in order to achieve this goal. These objective were: 1) to isolate and compare COX-1 and COX-2 inhibitory activities of alkamides from *E. purpurea* roots; 2) to evaluate the mosquitocidal activity of *E. purpurea* root alkamides; 3) to isolate flavonoids and anthocyanins from *E. purpurea* flowers; 4) to characterize of the COX-1 and COX-2 inhibitory activities and antioxidant activities of the flavonoids and anthocyanins from *E. purpurea* flowers; and 5) to characterize and examine the effects of purified constituents from *E. purpurea* roots and flowers (alkamides, flavonoids, and anthocyanins) on

cytokine production. The following literature review focuses on the chemical constituents and biological activities of extracts and compounds from of *Echinacea* spp., with emphasis on those obtained from *E. purpurea*.

CHAPTER 1. LITERATURE REVIEW

The genus *Echinacea* is composed of nine *Echinacea* species that are native to central and eastern North America. Only *Echinacea purpurea, Echinacea angustifolia*, and *Echinacea pallida* are of significant phytoceutical interest based on historical use, availability, and biological activity. A large amount of information has been generated regarding the biological activity of *Echinacea* spp., primarily as crude extracts (the usual form available to consumers), or in some cases, as pure compounds isolated from *E. purpurea* or *E. angustifolia*. This review summarizes the available literature regarding *Echinacea* species, with specific attention given to the botany, chemistry, and biological activities of *E. angustifolia*, *E. pallida*, and *E. purpurea*.

Botany of *Echinacea* species

Echinacea purpurea (Asteraceae), also known as the purple coneflower, is a perennial plant that grows to a height of approximately two to three feet (Foster and Duke, 1990). *Echinacea* derives its name from the Greek word, "echinos," ("hedgehog" or "sea urchin") referring to the round, spiny seedhead (Kindscher, 1989; Gunning, 1999). Although *Echinacea* species are grown for phytoceutical purposes, their commercial potential extends to the use of *Echinacea* as popular ornamentals in gardens (Li, 1998: Brown, 1986) and for cut flowers (Li, 1998; Starman et al., 1995). *Echinacea* plants grown from seed generally flower in two to three years during the months of June and July in the central United States

(Kindscher, 1989). Flowering of *E. purpurea* may be induced artificially in a greenhouse environment (Runkle et al., 1998).

Echinacea purpurea plants possess straight, slightly pubescent stalks terminating with a single flower (Hunter, 1995). Flowers have a center disk that is orange (Hunter, 1995) and bristled (Foster and Duke, 1990), from which reddish-purple to pink rays droop (Gleason and Cronquist, 1991). The flowers of *E. purpurea* differ from those of *E. angustifolia* by the deeper purple hue, as well as the broader rays of the flower itself (Hunter, 1995). The leaves of *E. purpurea* are lance-shaped to elliptical (Gleanson and Cronquist, 1991), coarsely toothed (Foster and Duke, 1990), and extend approximately 15 cm, 1.5-5 times in length as compared to width (Gleason and Cronquist, 1991). The roots of *E. purpurea* consist of a fibrous-rooted crown, ranging from simple to branched (Gleason and Cronquist, 1991). *Echinacea purpurea* is primarily found in prairie regions of the United States, extending from Texas to North Dakota and from Colorado to Kansas (Foster and Duke, 1990). *Echinacea purpurea* may also be found irregularly in Michigan, Kentucky, Tennessee, and Georgia (Gleason and Cronquist, 1991).

Echinacea angustifolia and *E. purpurea* are winter-hardy, heat-tolerant, and drought-resistant. *E. purpurea* is less dependent upon soil pH and moisture content than *E. angustifolia* (Li, 1998). Although native only to the Great Plains region (Foster and Duke, 1990), cultivation has resulted in the widespread availability of *Echinacea* for medicinal purposes. *Echinacea* roots, which are used to the greatest extent in commercial preparations, are generally harvested

in the third or fourth year (Li, 1998). Growth parameters have been altered to experimentally increase the yield of root material for phytoceutical preparations (Trypsteen et al., 1991).

Historical use of *Echinacea* spp.

Currently, seven of the top ten most used botanicals in the United States are based on plants used medicinally by Native Americans (Borchers et al., 2000). *Echinacea angustifolia* was one of the predominant species used by Native American Indians for the treatment of a variety of ailments. *Echinacea* roots were used externally to treat burns, insect bites, and wounds and internally to alleviate the pain of toothaches, headaches, chills, stomach cramps, and coughs (Bauer and Wagner, 1991). The tribes that used *Echinacea* included the Cheyenne, Choctaw, Dakota, Delaware, Fox Kiowa, Montana, Omaha Pawnee, Ponca, Sioux, and Winnebago (Borchers et al., 2000). Teas were used to treat rheumatism, arthritis, mumps, and measles (Kindscher, 1989). Roots were most often used; however, the juice squeezed from whole *Echinacea* plants and infusions brewed from fresh herb were also used, but to a lesser extent (Bauer and Wagner, 1991).

Early American settlers observed *Echinacea* use among Native Americans for the treatment of various ailments, and adopted its use for similar medical conditions. In addition, new uses were developed for *Echinacea* spp. including the treatment of syphilis. American settlers also used *Echinacea* externally on horses as a treatment for saddle sores (Bauer and Wagner, 1991). *Echinacea*

angustifolia was popularized among American settlers in 1871 by H. C. F. Meyer, who marketed a tincture of *E. angustifolia* as "Meyer's Blood Purifier" (Kindscher, 1989; Foster, 1985). Meyer's natural remedy was accompanied by exaggerated claims of miracle cures for snake bites, bee stings, 'mad dog' bites, tumors, eczema, gangrene, typhoid fever, mountain fever, malaria, and diphtheria (Bauer and Wagner, 1991: Kindscher, 1989). Studies performed in the early 1900's did not find any physiological activity, and *Echinacea* was determined to be ineffective for botulism, anthrax, rattlesnake venom, tetanus, septicemia, and tuberculosis. The lack of substantiation of medicinal properties of *Echinacea* aided in the decline of its popularity, and as a result *Echinacea* did not achieve widespread acceptance in the United States (Kindscher, 1989).

Echinacea preparations did not suffer the same fate in Europe, where they became popular in the early 1900s as a means to boost immunity and fight chronic infections (Bauer and Wagner, 1991). A great deal of research regarding the effectiveness of *Echinacea* has been performed in Europe, where laws regarding commercial availability and use of herbal supplements are much more liberal (Kindscher, 1989; Tyler, 1986). Based on the success enjoyed by *Echinacea* products in Europe, a resurgence in its popularity was seen in the United States in the late twentieth century. Today *Echinacea* is one of the top-selling herbal supplements in America (Gunning, 1999; Henry, 1999).

The popularity of *Echinacea* as a dietary supplement is due in part to its reported antiviral and nonspecific immunostimulatory properties (Borchers et al., 2000; Proksch and Wagner, 1987). The German Commission E Monographs

lists *E. purpurea* herb oral preparations as approved for respiratory tract infections, urinary tract infections, and colds. In addition, *E. purpurea* herb is approved for topical application to promote wound healing (Blumenthal, 1998; Percival, 2000).

Echinacea angustifolia and *E. pallida* herb preparations and *E. purpurea* and *E. angustifolia* root preparations have not been approved by the German Commission E (Percival, 2000). The lack of approval for these four *Echinacea* preparations is due to insufficient research or inadequate identification of the materials used in the trials (Percival, 2000). The therapeutic efficacy of commercial preparations from other *Echinacea* plant parts has not been clearly established (Turner et al., 2000; Barrett et al., 1999; Grimm and Müller, 1999; Cheminat et al., 1989). The bioavailability, potency, potential synergistic effects, and mechanisms of action of the biologically active components are unknown (Percival, 2000), leaving a great deal of research yet to be performed on *Echinacea* species.

Chemistry of *Echinacea* spp.

Echinacea angustifolia was the primary species used by Native American Indians for treating illnesses and injuries (Cheminat et al., 1989). Due to its commercial availability, *E. purpurea* has also become widely used in popular modern-day *Echinacea* herbal supplements. The literature generated in the last two decades reflects the inclusion of *E. purpurea* in biological testing. The

phytoceutical activity of mixtures of *E. angustifolia*, *E. purpurea*, and *E. pallida* are often reported.

Currently, *E. angustifolia*, *E. purpurea*, and *E. pallida* are of primary commercial and nutraceutical interest (Bauer and Foster, 1991; Bauer et al., 1988a), and similarities in phytoceutical effects and chemical profiles have been demonstrated (Bauer and Wagner, 1991). *Echinacea simulata* McGregor, *Echinacea paradoxa* (Norton) Britton, and *Echinacea tennesseensis* (Beadle) Small (a rare species located in the Cedar Glade region of Tennessee) have been evaluated for potential commercial use. These three species of *Echinacea* demonstrated similar biological activities and possessed chemical profiles like those of *E. angustifolia and E. pallida* (Bauer and Foster, 1991; Bauer et al., 1990). The incorporation of *E. simulata*, *E. paradoxa*, and *E. tennesseensis* into commercial natural product supplements is currently unlikely, given their lack of widespread, commercial availability.

Several classes of chemical compounds have been isolated from *Echinacea* species. Analysis of the roots, leaves, stems, and flower heads has resulted in the identification of a number of alkamides, caffeic acid derivatives, flavonoids, and polyacetylenes. These groups will be further reviewed.

Essential Oils

The essential oil of *Echinacea* spp. has been reported to contain α-humulene, borneol, germacra-4(15), 5E,10(14)-trien-1-β-ol, α-pinene, limonene, caryophyllene oxide, bornyl acetate, vanillin, β-pinene, carvomenthene, β-

caryophyllene, germacrene D, *p*-hydroxy-cinnamic acid methylester, β-farnesene, and myrcene (Bauer and Wagner, 1991). It has been suggested that *Echinacea* spp., due to their essential oil content rich in borneol and α-pinene, might find applications in the cosmetic industry (Li and Wang, 1998).

The essential oil content for the roots of *E. angustifolia* has been reported in the range of 0.04 to 0.1% (Bauer and Wagner, 1991), with ariel parts, flowering ariel parts, and dried leaves of *E. angustifolia* possessing less than 0.1% (Bos et al., 1988; Heinzer et al., 1988; Bauer and Wagner, 1991). The essential oil content of fresh *E. purpurea* roots has been reported to range from 0.005-0.22% (Bauer and Wagner, 1991), while the fresh herb (referring to the above-ground portion of the plant, whether flowering or not) ranges from 0.08-0.32%, and fresh flowerheads from 0.13-0.48% (Bauer and Wagner, 1991).

Germacra-4(15),5E,10(14)-trien-1β-ol, a sesquiterpene alcohol, was Isolated from fresh aerial parts of *E. purpurea* (Bauer et al., 1988d). Due to its volatile nature, it is not usually found in dried *Echinacea* plant materials. Capillary gas chromatography coupled with mass spectrometry has been used to identify over seventy volatile compounds present in the roots, flowers, stems, and leaves of chopped and ground *E. angustifolia*, *E. purpurea*, and *E. pallida*. *E. purpurea* volatile compounds have been summarized (Mazza and Cottrell, 1999) (**Table 1.1**).

Table 1.1. Volatile compounds from the headspace of *E. purpurea* tissues (adapted from Mazza and Cottrell, 1999).

	Root	Flower	Leaf	Stem
acetaldehyde	X	X	X	X
dimethyl sulfide	X	X	X	X
propanal			X	
2-methylpropanol	X	X		X
2-propenal	X			
2-butanone		X		
2-methylbutanol	X	X	X	X
3-methylbutanol	X	X	X	X
1-methylpropyl acetate	X			
trichloroacetic acid	X			
α-pinene	X	X	X	X
α-thujene			X	
geranyl acetate			X	X
camphene	X	X	X	X
hexanal	X	X	X	X
β-pinene	X	X	X	X
2-methyl-1-propanol	X			
sabinene/β-thujene	X	X	X	X
2-pentenal	X		X	
2-methyl-4-pentenal		X	X	X
β-myrcene		X	X	X
α-phellandrene	X			
α-terpinene	X	X	X	X
heptanal	X			
limonene	X	X	X	X
2-hexenal (cis)			X	
2-methyl-1-butanol	X			
3-methyl-1-butanol	X			
2-hexenal (trans)		X	X	X
ocimene	X	X	X	X
γ-terpinene		X	X	X
trans-ocimene		X	X	X
p-cymene	X	X	X	
hexyl acetate	X		X	
α-terpinolene		X	X	X
3-hexen-1-ol acetate			X	X
6-methyl-5-hepten-2-one	X			
1-hexanol	X	X	X	X
3-hexen-1-ol (trans)			X	
Allo-ocimene		X	X	X

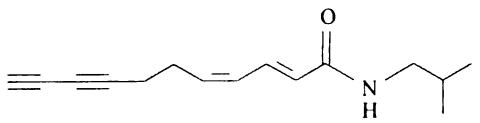
Table 1.1. Volatile compounds from the headspace of *E. purpurea* tissues (adapted from Mazza and Cottrell, 1999), continued.

	Root	Flower	Leaf	Stem
3-hexen-1-ol (cis)		X	X	X
2-hexen-1-ol (trans)		X		X
1-octen-3-ol	X			
benzaldehyde	X			
α-cubebene/α-copaene		X		
α-ylangene			X	X
γ-cadinene			X	
trans-caryophyllene		X	X	X
calarene			X	
germacrene D			X	X
5-ethyl-2(5H)-furanone			X	
δ-cadiene			X	X
βα-cubebene			X	X
2,2,3,3-tetramethyl hexane			X	

Alkamides

A number of alkamides have been isolated from *E. purpurea* roots (**Figure 1.1**). Two dodeca-2,4,8,10-tetraenoic acid isobutylamides, undeca-(2*Z*,4*E*)-dien-8,10-diynoic acid isobutylamide and dodeca-(2*Z*,4*E*)-dien-8,10-diynoic acid isobutylamide, were isolated from the roots of *E. angustifolia* and *E. purpurea* (Bohlmann and Grenz, 1966). *Echinacea angustifolia* roots yielded dodeca-(2*E*,4*E*)-dienoic acid and deca-(2*E*,4*E*,6*E*)-trienoic acid (Verelis, 1978; Bauer and Wagner, 1991). Trideca-(2*E*,7*Z*)-dien-10,12-diynoic acid, trideca-(2*E*,6*E*,8*Z*)-trien-10,12-diynoic acid and pentadeca-(2*E*,9*Z*)-dien-12,14-diynoic acid, trideca-(2*E*,7*Z*)-dien-10,12-diynoic acid (2-methylbutyl)amide, and pentadeca(2*E*,9*Z*)-dien-10,14-diynoic acid (2-hydroxyisobutylamide) were identified in the ariel parts of *E. purpurea* (Bohlmann and Hoffmann, 1983). An additional five alkamides from hexane extracts of *E. purpurea* roots were identified, including undeca-(2*E*,4*Z*)-dien-8,10-diynoic acid isobutylamide, dodeca-(2*E*,4*Z*)-dien-8,10-diynoic acid isobutylamide, dodeca-(2*E*,4*E*,10*E*)-trien-8-ynoic acid isobutylamide, dodeca-(2*E*,4*E*,8*Z*)-trienoic acid isobutylamide and dodeca-(2*E*,4*Z*)-dien-8,10-diyonic acid 2-methylbutylamide (Bauer et al., 1988b) (**Figure 1.1**).

Fifteen alkamides (**Figure 1.2**) were isolated from hexane extracts of *E. angustifolia* roots (Bauer et al., 1989). Undeca-(2*Z*)-en-8,10-diyonic acid isobutylamide, dodeca-(2*E*)-en-8,10-diyonic acid isobutylamide, undeca-(2*Z*)-en-8,10-diynoic acid 2-methylbutylamide, dodeca-(2*E*,4*Z*,10*Z*)trien-8-ynoic acid isobutylamide were identified in addition to previously isolated isobutylamides in *E. purpurea* roots (Bauer et al., 1989).



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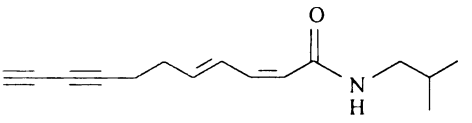

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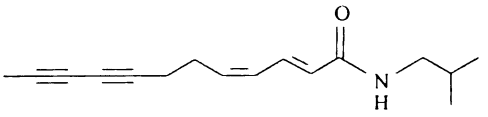

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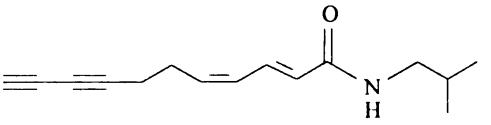
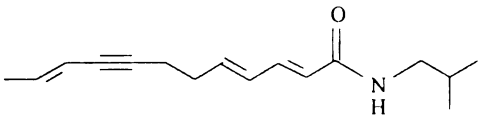
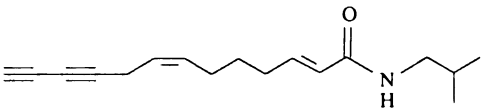


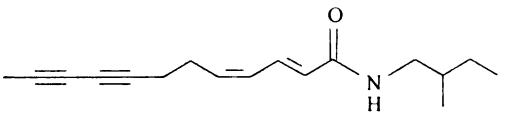
Undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide



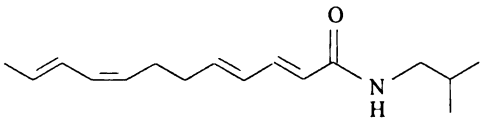
Dodeca-2*E*,4*E*,10*E*-trien-8-ynoic acid isobutylamide



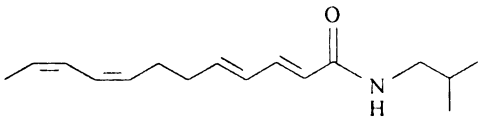
Trideca-2*E*,7*Z*-dien-10,12-diynoic acid isobutylamide



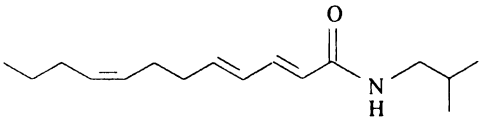
Dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methybutylamide



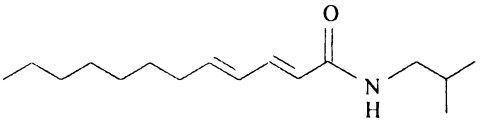
Dodeca-2*E*,4*E*,10*E*-tetraenoic acid isobutylamide



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

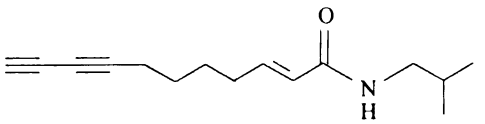


Dodeca-2*E*,4*Z*,8*Z*-trienoic acid isobutylamide

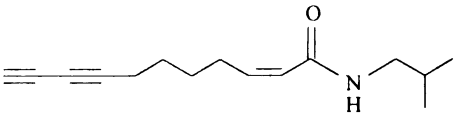


Dodeca-2*E*,4*E*-dienoic acid isobutylamide

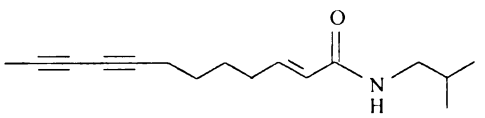
Figure 1.1. Alkamides from *E. purpurea* roots (Bauer and Wagner, 1991; Bauer et al., 1988b).



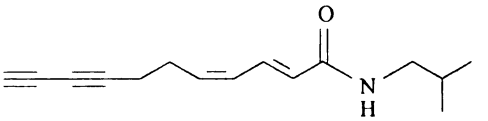
Undeca-2*E*-en-8,10-diynoic acid isobutylamide



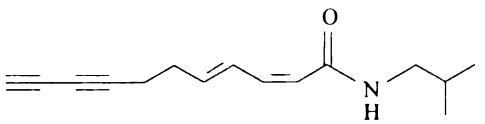
Undeca-2*Z*-en-8,10-diynoic acid isobutylamide



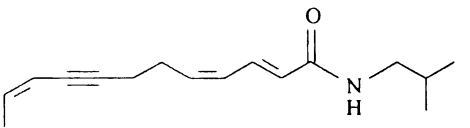
Dodeca-2*E*-en-8,10-diynoic acid isobutylamide



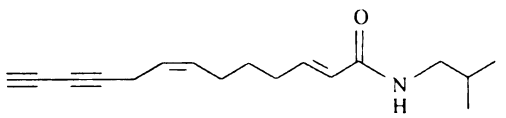
Undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide



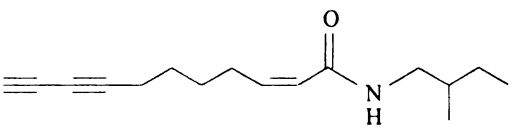
Undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutlyamide



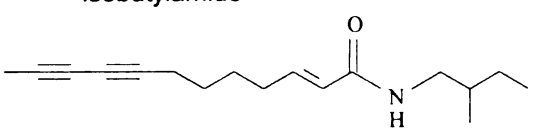
Dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide



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



Undeca-2*Z*-en-8,10-diynoic acid 2-methylbutylamide



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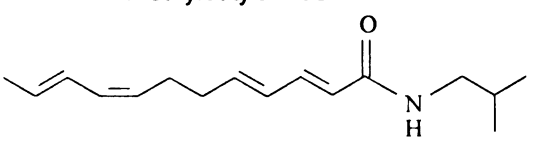
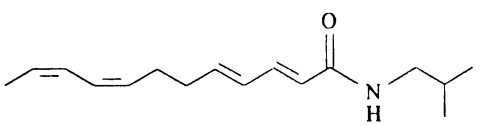
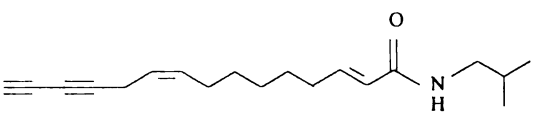


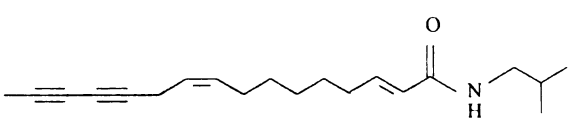
Dodeca-2*E*,4*E*,10*E*-tetraenoic acid isobutylamide



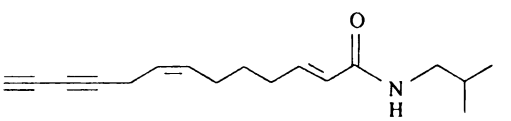
Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide



Pentadeca-2*E*,9*Z*-dien-12,14-dynoic acid isobuylamide



Hexadeca-2*E*,4*Z*-dien-12,14-diynoic acid isobutylamide



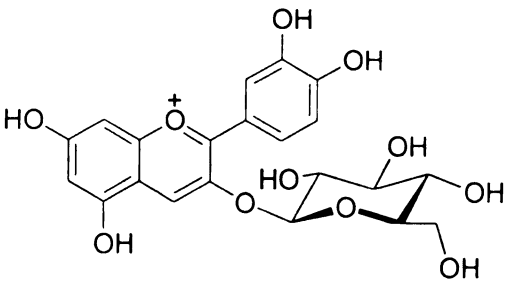
Pentadeca-2*E*,9*Z*-dien-12,14-diynoic acid isobutylamide

Figure 1.2. Alkamides from *E. angustifolia* roots (Bauer and Wagner, 1991; Bauer and Reminger, 1989).

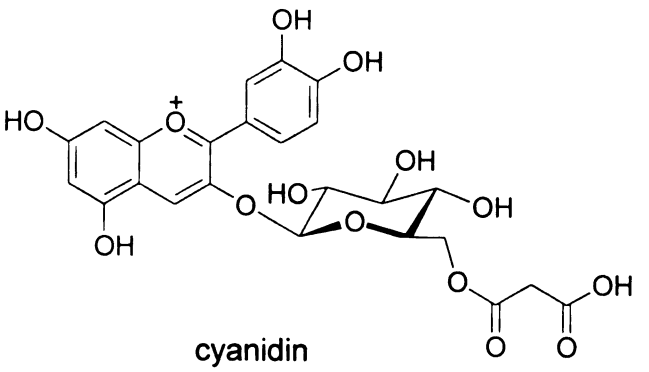
The development of pharmacologically active constituents from *Echinacea* spp. is a significant area of academic focus. Alkamides, which are known to be biologically active (Bauer and Wagner, 1991), are of particular interest. In an effort to understand the development of the alkamides found in *E. purpurea* and *E. angustifolia*, the germination of the achenes (fruits) was studied. Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide, the two primary alkamides isolated from adult plants (Bauer and Remiger, 1989), were found to be the main alkamides formed during germination of *E. purpurea* and *E. angustifolia* (Schulthess et al., 1991). Two additional isobutylamides found only in *E. purpurea*, trideca-2*E*, 7*Z*-diene-10,12-ciynoic acid isobutylamide, isolated from the achene and roots, and dodeca-2*E*,4*E*,10*E*-trien-8-ynoic acid isobutylamide, previously noted in the roots, were also found in the cotyledons. Levels of the two isobutylamides increased with primary leaf development (Schulthess et al., 1991).

Anthocyanins

Cyanidin 3-*O*-(*β*-D-glucopyranoside) and cyanidin 3-*O*-(6-*O*-malonyl-*β*-D-glucopyranoside) (**Figure 1.3**) were isolated from dry *E. purpurea* and *E. pallida* flowers by extraction with acetic acid-methanol-water followed by purification by ion exchange chromatography (Cheminat et al., 1989). Two additional acylated cyanidin glycosides were extracted from *E. purpurea*, however the structures were not fully elucidated (Cheminat et al., 1989).



cyanidin
3-O-β-D-glucopyranoside



cyanidin
3-O-malonyl-(1→6)-β-D-
glucopyranoside

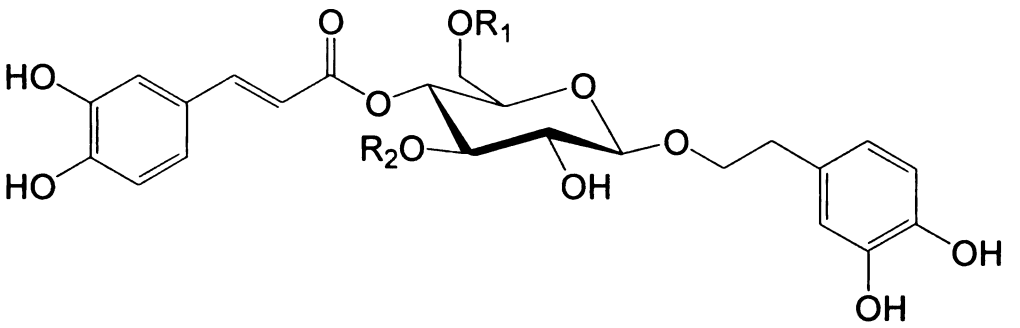
Figure 1.3. Anthocyanins isolated from *E. purpurea*.

Caffeic Acid Derivatives

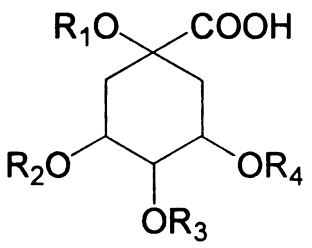
Caffeic acid derivatives (**Figure 1.4**) are considered to be among the most valuable and important active components of *Echinacea* spp. (Li and Wang, 1998). Cichoric acid (2,3-*O*-dicaffeoyl tartaric acid) was identified in *E. purpurea* flowers and roots at 1.2-3.1 and 0.6-2.1%, respectively (Bauer et al., 1988c). *Echinacea angustifolia* does not contain significant quantities of cichoric acid. Degradation of cichoric acid during Soxhlet extraction was suggested (Bergeron et al., 1999), based on the increased yield of cichoric acid when performing alcoholic, ultrasonic extraction of dried, ground roots.

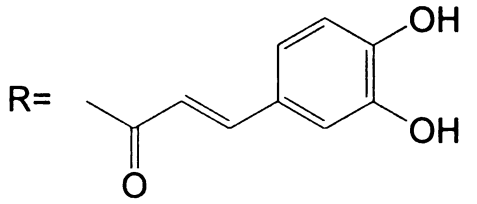
Echinacoside, (β-(3,4-dihydroxyphenyl)-ethyl-*O*-α-L-(1→6)-4-*O*-caffeoyl-β-D-glucopyranoside), has been identified in the alcoholic extracts of *E. angustifolia* and *E. pallida* roots (Bauer et al., 1988a), but has never been isolated from *E. purpurea* roots, allowing a distinction to be made between the species. Echinacoside has also been identified in the methanolic root extracts of *E. simulata* and *E. paradoxa* (Bauer and Foster, 1991). The quinic acid derivative, cynarine (1,5-*O*-dicaffeoyl quinic acid), is present in *E. angustifolia* but not *E. pallida*, providing a chromatographic marker to distinguish these species (Bauer et al., 1988c). Roots of *E. tennesseensis*, a rare species, do not contain echinacoside (Bauer et al., 1990).

Twelve caffeoyl derivatives have been isolated from *E. pallida* (Cheminat et al., 1988). Caffeic glycosides or caffeic esters of quinic or tartaric acid were identified from methanol extracts of *E. pallida* partitioned with ethyl acetate and *n*-butanol. Through preparative liquid chromatography and high performance



	R	R'
Echinacoside	Glucose (1,6-)	Rhamnose (1,3-)
6-*O*-Caffeoyl-echinacoside	6-*O*-Caffeoyl-glucose (1,6-)	Rhamnose (1,3-)
Verbascoside	H	Rhamnose (1,3-)
Desrhamnosyl-verbascoside	H	H

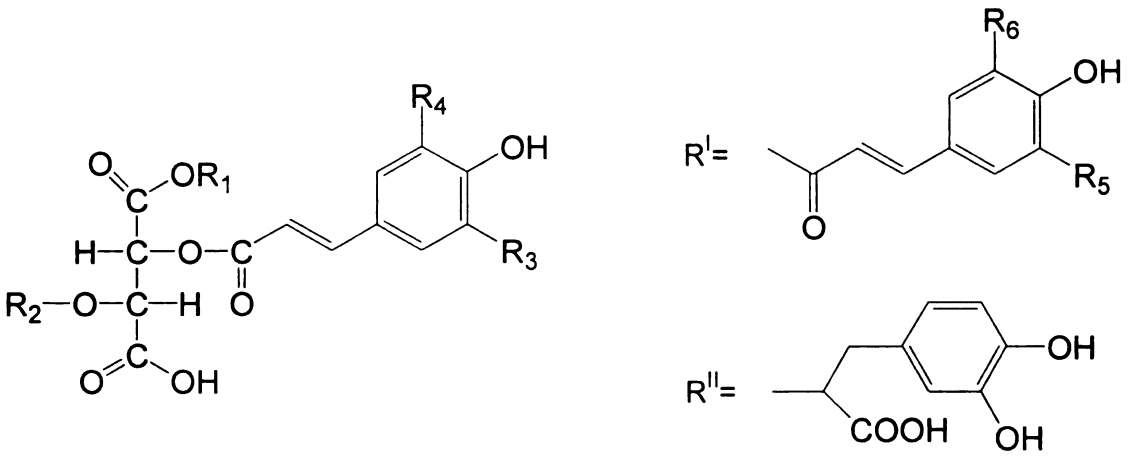




caffeic acid

	R_1	R_2	R_3	R_4
3-*O*-Caffeoyl-quinic acid (chlorogenic acid)	H	R	H	H
Isochlorogenic acids	H	R	R	H
	H	R	H	R
	H	H	R	R
Cynarine	R	H	H	R

Figure 1.4. Caffeic acid derviatives from *Echinacea* species (Bauer and Wagner, 1991; Cheminat et al., 1988).



	R_1	R_2	R_3	R_4	R_5	R_6
2-*O*-Caffeoyl-tartaric acid (Caftaric acid)	H	H	OH	H	-	-
2,3-*O*-Di-caffeoyl-tartaric acid (Cichoric acid)	H	R'	OH	H	OH	H
2,3-*O*-Di-caffeoyl-tartaric acid-methylester	CH_3	R'	OH	H	OH	H
2-*O*-Feruloyl-tartaric acid	H	H	OCH_3	H	-	-
2-*O*-Caffeoyl-3-*O*-coumaroyl-tartaric acid	H	R'	H	H	H	H
2-*O*-Caffeoyl-3-*O*-feruloyl-tartaric acid	H	R'	OH	H	OCH	H
2,3-*O*-Di-(5-[α-carboxy-β-(3,4-dihydroxy-phenyl)-ethyl]-caffeoyl)tartaric acid	H	R'	OH	R"	^{3}OH	R"
2-*O*-Caffeoyl-3-*O*-(5-[α-carboxy-β-(3,4-dihydroxy-phenyl)-ethyl]-caffeoyl)tartaric acid	H	R'	OH	H	OH	R"

Figure 1.4. Caffeic acid derviatives from *Echinacea* species (Bauer and Wagner, 1991; Cheminat et al., 1988), continued.

liquid chromatography, the following constituents were identified (Cheminat et al., 1988): 5-*O*-caffeoyl quinic acid (chlorogenic acid); 3,5-*O*-dicaffeoylquinic acid; 4,5-*O*-dicaffeoyl quinic acid; 2,3-*O*-dicaffeoyltartaric acid (cichoric acid); 2-*O*-caffeoyl-3-*O*-feruloyltartaric acid; 2-*O*-caffeoyltartaric acid (caftaric acid); 2-*O*-caffeoyl-3-*O*-5[α-carboxy-β-(3,4-dihydroxyphenyl)ethyl] caffeoyltartaric acid; 2,3-*O*-di 5-[α-carboxy-β-(3,4-dihydroxyphenyl)ethyl] caffeoyltartaric acid; β-(3,4-dihydroxyphenyl)-ethyl-*O*-4-*O*-caffeoyl-β-D-glucopyranoside; β-(3,4-dihydroxyphenyl)-ethyl-*O*-α-L-rhamnopyranosyl(1$\rightarrow$3)-4-*O*-caffeoyl-β-D-glucopyranoside (verbacoside); β-(3,4-dihydroxyphenyl)-ethyl-*O*-α-L-rhamnopyranosyl(1$\rightarrow$3)-β-D-glucopyranoside (1$\rightarrow$6)-4-*O*-caffeoyl-β-D-glucopyranoside (echinacoside); and β-(3,4-dihydroxyphenyl)-ethyl-*O*-α-L-rhamnopyranosyl (1$\rightarrow$3) (6-*O*-caffeoyl -β-D-glucopyranosyl) (1$\rightarrow$6)-4-*O*-caffeoyl-β-D-glucopyranoside (6-*O*-caffeoyl-echinacoside).

Solid-phase extraction coupled with reversed-phase HPLC has been used to isolate free phenolic acids for *E. purpurea*, *E. angustifolia*, and *E. pallida* for further study with regard to free radical scavenging (Facino et al., 1995) and increased efficiency of phenolic separation (Glowniak et al., 1996). The phenolic acid content of the dry, above ground portion of *Echinacea* species has been determined to range from 70 to 1400 μg/g (Glowniak et al., 1996).

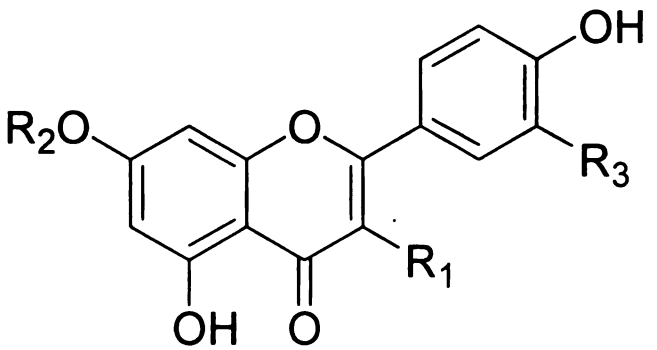
Flavonoids

Echinacea angustifolia leaves yielded the following flavonoids: luteolin, kaempferol, quercetin, quercetagetin-7-glucoside; luteolin-7-glucoside,

kaempferol-3-glucoside, quercetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-xyloside, quercetin-3-glucoside; kaempferol-3-rutinoside; rutoside; and isorhamnetin-3-rutinoside (Bauer and Wagner, 1991) (**Figure 1.5**). Quercetin, quercetin-7-glucoside, rutin, quercetin-3-robinobioside, and quercetin-3-xylosylgalactoside were identified in the leaves of *E. purpurea* (Bauer and Wagner, 1991) (**Figure 1.5**).

Polyacetylenes

A series of ketoalkynes and ketoalkenes have been isolated from *E. pallida*, including tetradeca-(8Z)-en-11,13-diyn-2-one, pentadeca-(8Z)-en-11,13-diyn-2-one, pentadeca-(8Z, 13A)-dien-11-yn-2-one, pentadeca-(8Z,11Z,13Z)-trien-2-one, pentadeca-(8Z,11E,13Z)-trien-2-one, pentadeca-(8Z,11Z)-dien-2-one, pentadeca-(8Z)-en-2-one and heptadeca-(8Z,11Z)-dien-2-one (Bauer et al., 1988a). Autoxidation occurred in stored materials, resulting in the formation of hydroxylated species. *Echinacea pallida* was mistaken for *E. angustifolia* in numerous preliminary studies (Bauer and Wagner, 1991), leading to a great deal of confusion regarding chemical constituents and biological activities assigned to *E. angustifolia*. Since *E. angustifolia* did not contain the above ketoalkynes and ketoalkenes, a distinction may be made between *E. pallida* and *E. angustifolia* based on constituent profiles.



Compound	R_1	R_2	R_3
luteolin	H	H	OH
apigenin	H	H	H
quercetin	OH	H	OH
kaempferol	OH	H	H
luteolin-7-glucoside	H	Glc	OH
kaempferol-3-glucoside	Glc	H	H
quercetin-3-arabinoside	Ara	H	OH
quercetin-3-galactoside	Gal	H	OH
quercetin-3-xyloside	Xyl	H	OH
quercetin-3-glucoside	Glc	H	OH
kaempferol-3-rutinoside	rutinose	H	H
isorhamnetin-3-rutinoside	rutinose	H	OCH_3
quercetin-7-glucoside	OH	Glc	OH
kaempferol-3-rutinoside	Glc	H	H
quercetin-3-robinobioside	robinobiose	H	OH

Figure 1.5. Flavonoids from *Echinacea* spp. (Bauer and Wagner, 1991).

Analytical methods for the identification of *Echinacea* constituents

Echinacea species have historically been misidentified in the preparation of extracts for scientific study and consumer use (Bauer and Wagner, 1991). Additionally, the adulteration of *Echinacea* extracts by the inclusion of other plant species has posed additional problems in the evaluation of the bioactivity of *Echinacea* extracts. To circumvent the confusion surrounding these problems, a number of analytical methods have been developed for the quality control and identification of chemical constituents in the roots, leaves, and flowers of *Echinacea* species (Lienert et al., 1998). Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Rogers et al., 1998; Bauer and Remiger, 1989; Bauer et al., 1988a) have been predominantly used to identify the characteristic compounds associated with *Echinacea* species. Gas chromatography coupled with mass spectrometry has been used to characterize the volatile constituents of *E. angustifolia*, *E. purpurea*, and *E. pallida* roots, leaves, stems, and flowers (Mazza and Cottrell, 1999) (**Table 1.1**), as well as to differentiate among the three species (Lienert et al., 1998). Isobutylamides are recognized as one of the most biologically active groups of compounds in *Echinacea* extracts (Bauer and Wagner, 1991). The rapid classification of *E. purpurea*, *E. angustifolia*, and *E. pallida*, based on isobutylamide content (Bauer and Remiger, 1989) and total phenolic content (Perry et al., 2001; Bauer et al., 1988a), has been made possible by HPLC analysis with UV detection.

Attempts to develop methods for the standardization of *Echinacea* extracts have suggested that *Echinacea* preparations might be characterized most

effectively by analysis of total phenolics (Perry et al., 2001; Bauer et al., 1988a). Cichoric acid is the primary phenolic compounds present in *E. purpurea* root and herb extracts, yet is absent from *E. angustifolia* (Perry et al., 2001; Bauer and Wagner, 1991). Echinacoside is the predominant phenolic compound in *E. pallida* and *E. angustifolia*, but is not found in *E. purpurea* (Perry et al., 2001; Bauer and Wagner, 1991). Research has focused on reverse-phase high performance liquid chromatography for the separation of phenolics in order to characterize *Echinacea* spp. (Bauer et al., 1988a). Marked differences in the distribution of characteristic constituents among species have been noted (Perry et al., 2001; Bauer et al., 1988a; Perry et al., 1997). A combination of solid-phase extraction and reverse phase HPLC for the analysis of *Echinacea* spp. has shown success for the rapid screening of phenolic acid content (Glowniak et al., 1996).

The rapid classification of *E. purpurea*, *E. angustifolia*, and *E. pallida* based on isobutylamide content (Bauer and Remiger, 1989) was examined through TLC. *Echinacea pallida* root extracts contained almost no amides, while *E. purpurea* and *E. angustifolia* extracts showed the presence of numerous alkamides characteristic to *Echinacea* species (Bauer and Remiger, 1989). Reverse phase HPLC coupled with electrospray mass spectrometry was used to analyze the alkamides in *E. purpurea* roots (Sloley et al., 2001;He et al., 1998).

Knowing the exact chemical profile and having a standard method by which to determine the presence of characteristic constituents would allow nutraceutical/phytoceutical manufacturers to more easily identify and compare

the starting materials for medicinal preparations (Sloley et al., 2001). Although optimization of HPLC-UV methods and extraction schemes have aided in attempts to characterize markers to distinguish between *Echinacea* spp., disparity within each species suggested large variations in the raw materials used for *Echinacea* preparations (Bergeron et al., 2000). The similarities among plant species, particularly between *E. angustifolia* and *E. pallida*, have occasionally caused confusion regarding the proper identity of plants used for nutraceutical preparations (Sloley et al., 2001). The chemical fingerprinting of *Echinacea* extracts can mitigate which species was used in the preparation as well as indicate the portion of the plant extracted (Perry et al., 2001).

Stability of *Echinacea* constituents

A serious question regarding the efficacy of nutraceuticals is whether active components are stable enough in *Echinacea* preparations to convey biological activity to the consumer. Of particular interest are alkamides and phenolic acids (Bauer and Wagner, 1991). Alkamides are believed to be susceptible to degradation upon storage at various temperatures (Perry et al., 2000; Kim et al., 2000a; Rogers et al., 1998), as a result of an autoxidative process (Perry et al., 2000).

Cichoric acid, one of the major compounds in the fresh herb of *E. purpurea*, has numerous documented biological effects (Nüsslein et al., 2000), yet its susceptibility to enzymatic degradation during the processing of fresh *E. purpurea* plants makes the preparation of *E. purpurea*-based phytoceuticals a

challenge. Cichoric acid is rapidly degraded by polyphenol oxidase in fresh plant juices, however might be preserved to some extent by the addition of ethanol combined with an antioxidant such as ascorbic acid to inhibit enzyme activity (Nüsslein et al., 2000). Drying *E. purpurea* flowers by freeze-drying, vacuum microwave drying, and air-drying drastically reduced the cichoric acid and caftaric acid quantities (Kim et al., 2000b).

The chemical content of stored plant materials and various preparations has been examined to determine the potential for degradation of alkamides (Bauer and Wagner, 1991). Although many plants are air-dried, the levels of active constituents might be altered during the process, and the procedure is time-consuming (Kim et al., 2000a). The alkamide content of *E. purpurea* roots was unaffected by drying in an oven at 32-33°C when roots were broken into large pieces or when chopped into one centimeter pieces to speed the drying process (Perry et al., 2000). This method of drying did not reduce the alkamide content of *E. purpurea* roots, indicating that oven-drying might be a suitable alternative for more rapid processing of *Echinacea* root material. In a comparison of freeze-drying, vacuum microwave drying, and air-drying, freeze-drying was found to be the best method to preserve alkamides in *E. purpurea* roots (Kim et al., 2000a).

Although not significantly altered by oven-drying at 32-33°C (Perry et al., 1999), alkamide levels drop over time based on storage temperature (Perry et al., 2000; Rogers et al., 1998). Samples of powdered *E. angustifolia* roots stored at 25°C in a dessicator lost 13% of their alkamide contents over a two month

period (Rogers et al., 1998). The alkamide contents of roots, either chopped or whole, stored at 24°C was ≤20% of the roots stored at –18 °C for sixteen weeks (Perry et al., 2000). Unfortunately, the initial alkamide content of freshly dried *E. purpurea* root was not assessed at the start of the study, making only relative comparisons of alkamide content possible rather than indicating absolute loss. Storage at -18°C for sixty-four weeks further reduced alkamide content by 60% when compared to storage at -18°C for sixteen weeks (Perry et al., 2000).

Biological activity of *Echinacea* spp. extracts and constituents

Numerous biological activities have been reported for extracts and compounds from *Echinacea* spp., including the stimulation of immune function, anti-viral effects, antioxidant activity, and anti-inflammatory activity. Clinical studies, performed predominantly in Germany, have analyzed the effects of various *Echinacea* preparations used for the treatment of colds and upper respiratory tract infections, recurrent vaginal *Candida* infections, and for acute bronchitis in children (Bauer, 1998). Dosing remains a problem, since effective doses and active constituents have not been identified. Currently, traditional dosing of *Echinacea* products is based on 6-9 mL of expressed juice of the plant, 1.5-7.5 mL of tincture, or 2-5 g of dried root per day (Zink and Chaffin, 1998). Of the chemical constituents isolated from *Echinacea*, caffeic acid derivatives, alkylamides, polysaccharides, and polyacetylenes are thought to be among the most biologically active (Li and Wang, 1998).

Toxicity of Echinacea spp.

Echinacea has been used in various medicinal preparations for hundreds of years (Bauer and Wagner, 1991). In general, the use of *Echinacea* preparations is perceived to be safe given the lack of reported widespread problems (Parnham, 1996). Concern for individuals who may take multiple medications, particularly the elderly, has raised the question of the possibility of drug-herb interactions (Ness et al., 1999). Individuals taking immunosuppressants, such as corticosteroids and cyclosporine, should not use *Echinacea* products, since *Echinacea* is believed to be an effective immunostimulant (Miller, 1998). Additionally, it has been suggested that use of *Echinacea* should not exceed eight weeks due to the potential for hepatotoxicity, and should not be used with known hepatoxic drugs such as anabolic steroids, amiodarone, methotrexate, and ketoconazole (Miller, 1998). *Echinacea* is also not recommended for individuals with autoimmune disorders and progressive diseases, including tuberculosis, AIDS, multiple sclerosis, leukosis, collagen disorders, or diabetes mellitus (Miller, 1998). Although generally regarded as safe due to the sheer numbers of individuals taking *Echinacea* preparations and lack of reported adverse effects (Borchers et al.,2000), studies have been performed to examine the potential for toxicity and adverse reactions.

Echinacea extract injections have been reported to cause transient tachychardia and influenza-like symptoms in humans (Borchers et al., 2000; Miller, 1998; Jurcic et al., 1989). Oral administrations have yielded slightly higher adverse reactions compared to placebo, including headache, nausea, and

fatigue (Schmidt et al., 1990). An increase in body temperature by 0.5 to 1.0°C has also been reported to occur with the use of *Echinacea* preparations (Bauer, 1998). *Echinacea angustifolia* preparations have been associated with more adverse effects than *E. purpurea* (Melchart et al., 1998).

Adverse reactions to the oral use of *Echinacea* expressed juice are considered to be rare (Bauer, 1998), although acute allergic reactions are possible (Lersch et al., 1992). One report of *Echinacea*-associated anaphylaxis has been reported in an individual who commonly consumed *Echinacea* preparations (Mullins, 1998). It should be noted that the individual also consumed a variety of vitamins and natural product supplements with the *Echinacea*, and exceeded the manufacturer's recommended daily dose by consuming twice the recommended amount. While testing negative in skin-prick tests for reactivity to various allergen extracts, the individual tested positive for the ingested *Echinacea* preparation. Testing of *Echinacea* preparations in eighty-four patients resulted in 19% (sixteen subjects) of subjects yielding a strong positive reaction, even though only two of the subjects had ingested *Echinacea* preparations previously (Mullins, 1998). The results suggest cross-reactivity to structurally related proteins common to *Echinacea*, and would appear to indicate that even the first-time use of *Echinacea* preparations could result in allergic reactions (Mullins, 1998).

The lack of any other reported cases of *Echinacea*-related anaphylaxis indicates that the threshold used to indicate cross-reactivity might have been too low in the previous study (Borchers et al., 2000; Myers and Wohlmuth, 1998). A

component in the *Echinacea* preparation, not the *Echinacea* juice, has been suggested as the cause of the allergenic response (Myers and Wohlmuth, 1998). Given the lack of reported cases of *Echinacea*-related allergenic response and the number of people consuming *Echinacea* herbal supplements, concerns regarding severe allergenic response are not warranted. As a result, *Echinacea* is considered to be safe and well-tolerated (Borchers et al., 2000).

In vivo testing of expressed juice of *E. purpurea* did not demonstrate toxicity in rats and mice following four weeks of oral administration (Mengs et al. 1991). Acute toxicity was investigated through the single dosing of *Echinacea* juice, either intravenous (15 g/kg) or oral (5 g/kg), to groups of sixteen Wistar rats and sixteen NMRI mice (eight males/eight females per group) for each dosing method. No significant reactions occurred, and no changes to organs were noted upon necropsy (Mengs et al., 1991)

A subacute toxicity study was designed to assess the safety of *Echinacea* preparations. Eighteen male and eighteen female Wistar rats were given 0, 800, 2400, or 8000 mg/kg body weight/day of expressed *E. purpurea* juice for four weeks. The two highest doses showed a significant lowering of plasma alkaline phosphatase in males, and a rise in prothrombin time in females. No relevant differences in weight, food consumption, necropsy, or histology results were noted (Mengs et al., 1991).

The mutagenic potential of *E. purpurea* extracts was evaluated using several *Salmonella typhimurium* strains and in L5178Y mouse lymphoma cells. No evidence of mutagenicity was noted in either the bacterial or mammalian

mutation assay (Mengs et al., 1991). Examination of *E. purpurea* extracts on human lymphocytes yielded no notable evidence of toxicity. Combined with the above negative results for acute or subacute toxicity, *E. purpurea* preparations are regarded as non-toxic (Mengs et al., 1991); however, no tests for epigenetic toxicity were conducted.

Antimicrobial properties of Echinacea spp.

Bactericidal or bacteriostatic activities have not been demonstrated by *Echinacea* extracts (Zink and Chaffin, 1998). Based upon the use of *Echinacea* as a topical treatment for alleviation of various infections, sore throat, sore gums, and infected wounds, the antifungal activity of *E. purpurea* was examined to determine the effectiveness of polyacetylenes and alkyamides on fungal growth. Tea brewed from *E. purpurea* and extracts prepared from *E. purpurea* herb were not active; however, extracts prepared from *E. purpurea* roots and commercially available tinctures, combinations of herb and roots of both *E. purpurea* and *E. angustifolia*, demonstrated antifungal activity (Binns et al., 1991). Pure compounds isolated from *E. purpurea* roots, including undeca-2*E*,4*Z*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, undeca-2*E*,4Z-diene-8,10-diynoic acid isobutylamide, and trideca-1-ene-3,5,7,9,10-pentayne, were found to possess antifungal activity proportional to the number of triple bonds present (Binns et al., 1991). Evaluation of antimicrobial activity lends support to anecdotal claims for the topical use of *Echinacea* preparations for certain conditions.

Effects of Echinacea on glucose metabolism

In recent years, research interest in the effects of herbal supplement use on glucose metabolism in humans has increased. Potential nutraceutical treatments include *Mormordica charantia* (bitter melon), *Ocimum sanctum* (holy basil), *Allium cepa* (onion), and *Allium sativum* (garlic), which have demonstrated hypoglycemic effects in individuals with diabetes (Broadhurst et al, 2000; Srivastava et al., 1993; Rai et al., 1997; Koch and Lawson, 1996). In light of this interest, ammonium hydroxide extracts of *E. purpurea* roots were investigated for in vitro effects on insulin-dependent glucose metabolism in rat epididymal adipocytes. *E. purpurea* root extracts did not show any significant activity (Broadhurst et al., 2000).

Potential reproductive effects of Echinacea products

Potential detrimental effects of herbal supplements on reproductive function were assessed to determine effects on sperm DNA and the fertilization process (Ondrizek et al, 1999). In order to simulate serum or semen concentrations, *Echinacea* was tested at 0.8 mg/mL and 8 mg/mL, with the low dose representative of one 1/1000th of the recommended daily dose of the herbal extract. The high dose (8mg/mL) damaged sperm DNA, reduced sperm viability, and reduced oocyte penetration. Reduction in oocyte penetration by treatment with *Echinacea* was suggested to be due to an inhibition of the hyaluronidase enzyme in the sperm head, rather than an effect on sperm motility (Ondrizek et al., 1999). While the level of *Echinacea* extract and/or components

in semen is currently unknown, the results indicate the potential for adverse reproductive effects.

Immune-stimulating properties of Echinacea spp.

4-*O*-Methyl-glucuronoarabinoxylan, isolated from *E. purpurea*, is one of the polysaccharides believed to be responsible for enhancing phagocytosis effects in an in vitro granulocyte assay (Proksch and Wagner, 1987). Three additional polysaccharides, an acidic arabinogalactan and two fucogalactoxyloglucans with mean molecular weights of 10,000 and 25,000, were identified in *E. purpurea* cell cultures (Wagner et al., 1988). The acidic arabinogalactan stimulated macrophages to excrete tumor necrosis factor, and the higher molecular weight fucogalactoxyglucan enhanced phagocytosis in vitro and in vivo (Wagner et al., 1988).

It has been reported that *Echinacea* whole plant extracts possess antiviral activity (Wacker and Hilbig, 1978). The aqueous and methanol crude extracts of *E. purpurea* were assayed for resistance to herpes, influenza, and vesicular stomatitis viruses, and were found to provide 50-80% resistance to the viruses upon incubation with the extracts (Wacker and Hilbig, 1978). Echinacoside, caffeic acid, and cichoric acid were examined in a vesicular stomatitis virus assay using mouse L-929 cells (Cheminat et al., 1988). Incubation of vesicular stomatitis virus with 62.5 μg/ml caffeic acid or 125 μg/ml of cichoric acid for four hours reduced the infectivity of the virus more than 50% (Cheminat et al., 1988).

The immunostimulating properties of acidic arabinogalactan produced by *E. purpurea* cell cultures, molecular weight 75,000, were examined to determine effectiveness in activating macrophages against *Leishmania enriettii* and tumor cells (Luettig et al., 1989). The acidic arabinogalactan decreased *L. enriettii* growth in intracellular and phagocytosis-associated assays. Macrophages were induced by arabinogalactan to produce tumor necrosis factor, interleukin-1, and interferon-β_2, however B cells and T cells were unaffected.

Antigen specificity to antibodies raised against *E. purpurea* was determined by Egert and Beuscher (1992). Antisera raised against *E. purpurea* were tested using an enzyme-linked immunosorbent assay (ELISA) developed to determine the presence of glycoproteins and polysaccharides. Polymer antigens from *Baptisia tinctoria*, *Thuja occidentalis*, lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, and LPS from *Salmonella typhimurium* did not cross-react with antibodies raised against *E. purpurea* (Egert and Beuscher, 1992).

Antioxidant Properties

Natural antioxidants are increasing in popularity due to their reported role as modulators of lipid peroxidation as it relates to diseases such as atherogenesis, thrombosis, and carcinogenesis (Frankel, 1999). Phenolic antioxidants are known to have free radical scavenging properties and antioxidant properties with regard to the oxidation of low-density lipoproteins. *Echinacea* spp. possess a variety of phenolic antioxidants (Bauer and Wagner,

1991) that are believed to be responsible for much of the antioxidant activity of the plant extracts (Facino et al., 1995).

The ability of caffeoyl derivatives from *Echinacea* species to prevent free radical-induced degradation of collagen has been assessed (Facino et al., 1995). Caffeic acid, chlorogenic acid, cichoric acid, cynarine, and echinacoside were assayed in a model system measuring collagen degradation in the presence of xanthine oxidase, with and without iron as an oxidation catalyst. Protective effects were noted for all species, however cichoric acid and echinacoside were most active (Facino et al., 1995).

The total antioxidant activity of a phenolic *Echinacea* extract, standardized to 4.5% echinacoside content, was assessed at 0.5 mg/ml by the Trolox equivalent antioxidant activity method (Pietta et al., 1998). The Trolox equivalent antioxidant activity reflects the ability of an antioxidant to scavenge a radical cation ABTS+ - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate). The *Echinacea* extract was determined to be ineffective (Pietta et al., 1998). It should be noted that the source of the *Echinacea* extracts was not clearly indicated (i.e. root, herb, or flower). Additional studies were performed to assess the antioxidant activity of *E. purpurea, E. angustifolia, and E. pallida* root and leaf extracts in a hydroxyl free radical scavenging assay that measures the peroxidase-catalyzed accumulation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sloley et al., 2000). Root and leaf extracts of all three species demonstrated hydroxyl free radical scavenging activity, with *E. purpurea* root extracts showing higher activity than *E. angustifolia* or *E. pallida*.

The ability of *E. purpurea*, *E. angustifolia*, and *E. pallida* root and leaf extracts to inhibit Fe^{2+}-induced lipid peroxidation was assessed in a catecholaminergic neuroblastoma SH-SY5Y cells using a thiobarbituric acid assay system (Sloley et al., 2000). The leaf extracts of all three *Echinacea* spp. demonstrated higher antioxidant activity than root extracts. No significant differences were observed between the leaf extracts of the three *Echinacea* species. Chemical profiles, determined by HPLC analysis, were distinctly different for the leaf extracts of the three species suggesting that multiple constituents might be responsible for the antioxidant activity (Sloley et al., 2000).

Methanol extracts of lyophilized, ground *E. purpurea*, *E. angustifolia*, and *E. pallida* roots were investigated in several model systems to elucidate the antioxidant activity of *Echinacea* spp. (Hu and Kitts, 2000). Reducing power, evaluated using $K_3Fe(CN)_6$-$FeCl_3$ and determined by comparison to ascorbic acid, and copper chelating capacity measured by UV-vis spectrophotmetry, were highest for *E. pallida*. *Echinacea* extracts suppressed hydroxyl radical generation by 20-34% in a concentration dependent manner, and *E. pallida* was superior to *E. angustifolia* and *E. purpurea* in free radical scavenging activity (Hu and Kitts, 2000).

Anti-inflammatory Properties

The anti-inflammatory properties of *E. angustifolia* root extracts were evaluated in the carrageenan paw edema and croton oil dermatitis assays (Tragini et al., 1985). Intraveneous administration of aqueous *E. angustifolia* root

extracts inhibited carrageenan-induced paw inflammation. In another test, an *E. angustifolia* root extract was applied to the ears of mice simultaneously with croton oil as an irritant. The tests revealed that *E. angustifolia* exhibited anti-inflammatory activity that was superior compared to both negative and positive (benzidamine) controls. Further examination indicated that a polysaccharide fraction of *E. angustifolia* roots was responsible for the effects demonstrated in the carrageenan paw edema and croton oil ear test (Tubaro et al., 1987). Higher molecular weight fractions of *E. angustifolia* root polysaccharide extracts, separated by tangential flow filtration, also demonstrated activity in the croton oil ear test (Tragni et al., 1988).

Eight polyunsaturated alkamides from *E. angustifolia* were tested for inhibition of cyclooxygenase and 5-lipoxygenase, two enzymes of the arachiconic acid metabolic pathways (Muller-Jakic et al., 1994). The *n*-hexane extract of *E. angustifolia* roots, the major component of which was a mixture of isomers, doceca-2*E*,4*E*,8Z,10E/Z-tetraenoic acid isobutylamides, inhibited both cyclooxygenase and 5-lipoxygenase. Other isobutylamides tested included dodeca-2*E*,4Z,10Z-triene-8-ynoic acid isobutylamide, dodeca-2*E*-ene-8,10-diynoic acid isobutylamide, pentadeca-2*E*,9Z-diene-12,14-diynoic acid isobutylamide, hexadeca-2*E*,9Z-diene-12,14-diynoic acid isobutylamide, undeca-2*E*-ene-8,10-diynoic acid isobutylamide, undeca-2Z-ene-8,10-diynoic acid isobutylamide, and undeca-2Z-ene-8,10-diynoic acid 2-methylbutylamide. Pentadeca-2*E*,9Z-diene-12,14-diynoic acid isobutylamide and hexadeca-2*E*,9Z-diene-12,14-diynoic acid isobutylamide showed little activity in the lipoxygenase

assay; however, they did inhibit cyclooxygenase activity. Another alkamide, dodeca-2*E*,4*Z*,10*Z*-triene-8-ynoic acid isobutylamide, is structurally similar to the active alkamides, but it was active only at high concentrations to both enzymes. The competitive inhibition of 5-lipoxygenase and cyclooxygenase enzymes by alkamides acting as arachidonic acid analogues has been suggested (Muller-Jakic et al., 1994).

Clinical studies denoting the effectiveness of *Echinacea* for treating upper respiratory tract infections

Echinacea has been tested in a number of clinical studies to determine its effects on relieving the severity and reducing the duration of upper respiratory tract infections. In one study, 160 subjects were given either a placebo or 900 mg of *E. pallida* root extract daily for eight to ten days. The results indicated that treatment with *Echinacea* significantly reduced the duration of the respiratory tract infection from 13 to 9.8 days (Bräunig et al., 1993). A follow-up study was designed to examine dose-response response in which 180 subjects were given a placebo, 900 mg per day, or 450 mg per day of *E. purpurea* root extract. Evaluation of cold symptoms at 3-4 and 8-10 days indicated that only the high-dose of *E. purpurea* significantly reduced symptoms (Gunning, 1999; Bräunig et al., 1992).

In another study a dried, ethanol extract of *E. purpurea* (95% herb and 5% root) in tablet form, marketed as Echinaforce™, was administered three times daily (two tablets per dose) for eight days (Brinkeborn et al., 1998). The severity

of a variety of symptoms was evaluated in 119 subjects on either day one or two and again on day eight after the onset of an upper respiratory tract infection. A statistically significant level of improvement was demonstrated for the treatment group when compared to the placebo group (Brinkeborn et al., 1998).

The effects of Echinagard™ (also known as Echinacin™), a commercial product made from the juice of *E. purpurea* herb, were assessed in a double-blind randomized placebo-controlled clinical trial of 120 participants. At the first indication of an upper respiratory tract infection, subjects ingested 20 drops every two hours on day one, then three times per day on each subsequent day until symptoms resolved (Hoheisel et al., 1997). According to the results, a significant number of subjects in the placebo group, 60%, compared to the Echinagard™-treated group, 40%, developed an upper respiratory tract infection (Hoheisel et al., 1997).

Clinical studies failing to demonstrate the effectiveness of *Echinacea* for treating upper respiratory tract infections

Many clinical trials have indicated that there are no significant effects of *Echinacea* extracts on the duration or severity of colds and respiratory tract infections (Bauer, 1998; Gunning, 1999). For example, a study involving 108 patients with a prior history of upper respiratory tract infections (defined as more than three upper respiratory tract infections in the previous six months), examined the effect of expressed *E. purpurea* juice administered at a dose of 4 mL twice daily for eight weeks. The *E. purpurea* juice did not significantly reduce

the number or duration of upper respiratory tract infections when compared to the placebo (Schöneberger, 1992).

A three-armed, randomized, double-blind trial with 302 subjects was designed to determine the effects of both *E. purpurea* and *E. angustifolia* root extracts in the prevention of upper respiratory tract infections. Subjects were dosed with 50 drops of a placebo, *E. purpurea* root extract, or *E. angustifolia* root extract twice daily for twelve weeks. No significant differences were found between the treatment groups with regard to the median time to the development of an upper respiratory tract infection. Lack of power was suggested as the reason why no significant differences were detected between treatment groups. A larger study population was suggested for additional trials involving *Echinacea* spp. (Melchart et al., 1998).

A double-blind clinical trial was designed to determine the prophylactic effect of expressed *E. purpurea* juice on the incidence, duration, and severity of colds and respiratory infections (Grimm and Müller, 1999). One hundred eight subjects (chosen due to a history of three or more colds/respiratory tract infections in the previous year) were given either 4 mL of *E. purpurea* juice or placebo twice daily for eight weeks. During the trial, 65% of patients treated with *Echinacea* and 74% of placebo-treated subjects developed at least one cold or respiratory tract infection; however, these results were not statistically different from one another. In addition, no significant differences were noted between the placebo-treated and *Echinacea*-treated groups with regard to the incidence,

duration, or severity of colds and respiratory tract infections (Grimm and Müller, 1999).

In another clinical trial, an *Echinacea* preparation, analyzed and found to contain 0.16% cichoric acid and no echinacosides or alkamides, was tested for its ability to prevent rhinovirus colds (Turner et al., 2000). One hundred seventeen subjects were treated three times a day for two weeks with either the *Echinacea* extract at a dose of 300 mg or with the placebo. After the two-week treatment period, subjects were challenged with rhinovirus type 23, and the treatments were continued for another five days. Rhinovirus infection was detected in 44% of the *Echinacea*-treated subjects and 57% of the placebo group. In addition, clinical colds developed in 50% of the rhinovirus-infected *Echinacea*-treated subjects and 59% of the placebo group. None of the reductions in the incidence or severity of symptoms reported in the treatment groups were significant (Turner et al., 2000).

Mixed commercial preparations used in clinical trials

Resistan™, a commercial preparation that has been used in several clinical trials, contains *E. angustifolia* herb and root in addition to extracts of *Eupatorium*, *Baptista*, and *Amica*. In one trial, 100 subjects within two days of an upper respiratory tract infection onset were treated with either 30 mL of a placebo or Resistan™ on days one and two, followed by 15 mL on days three through six. Sore throat, nasal drainage, and cough plus pharyngeal erythema were found to be significantly improved in the group receiving the Resistan™ versus the

placebo (Dorn, 1989). A similar trial of Resistan™ involved 100 participants administered the herbal preparation during the first two days of the onset of an upper respiratory tract infection (Vorberg and Schneider, 1989). Symptom scores recorded on days three and eight showed significant improvement among subject receiving Resistan™ versus placebo. A 20% improvement in symptom score was observed on day three compared to placebo that increased to 50% by day eight (Vorberg and Schneider, 1989). A third trial of Resistan™ examined the potential of the herbal supplement for the prevention, rather than treatment, of upper respiratory tract infections (Schmidt et al., 1990). A group of 646 college students was administered Resistan™ or a placebo daily for eight weeks. An assessment of symptoms including cough, sore throat, difficulty swallowing, nasal drainage, congestion, headache, muscle aches, and fatigue was made every two weeks. The *Echinacea* group demonstrated a trend of 15% lower frequency of infection than the placebo group (Schmidt et al., 1990).

Another commercial *Echinacea* preparation, composed of *E. purpurea* and *E. pallida* roots, *Baptisiae tinctoriae* roots, and *Thujae occidentalis* herb, was examined in a double-blind placebo-controlled study involving fifteen clinical centers. The purpose of the study was to determine if the herbal remedy would be effective for patients in initial stages of an upper respiratory tract infection (Henneicke-von Zepelin et al., 1999). A study group of 263 individuals seeking medical advice for an acute common cold were given either placebo tablets or Esberitox® tablets (composed of 2 mg of *Thujae occidentalis*, 3.25 mg of *Echinacea pallida*, 3.25 mg of *Echinacea purpurea*, and 10 mg *Baptisiae*

tinctoriae) or placebo. Subjects were dosed with three tablets for seven to nine days following the first visit to their physician. Based upon the scoring of rhinitis, bronchitis, and overall severity of the cold, the herbal remedy was considered to be significantly better than the placebo (Henneicke-von Zepelin et al., 1999).

Hypothesis

Immune enhancement and inflammation, pain, and fever relief are anecdotal claims associated with the use of *E. purpurea*. However, specific components responsible for these claims have not been completely identified. It is hypothesized that *E. purpurea* contains compounds that are responsible for the reported biological activity of this plant that can be identified through bioassay-guided fractionation. This hypothesis was examined by addressing five primary objectives: 1) isolate and compare the COX-1 and COX-2 activities of alkamides isolated from *E. purpurea* roots; 2) evaluate the cytotoxicity of *E. purpurea* root alkamides based upon mosquitocidal activity; 3) isolate flavonoids and anthocyanins from *E. purpurea* flowers; 4) characterize the COX-1 and COX-2 activities and antioxidant activities of the flavonoids and anthocyanins isolated from *E. purpurea* flowers; and 5) characterize and examine the effects of purified constituents from *E. purpurea* roots and flowers (alkamides, flavonoids, and anthocyanins) on cytokine production. The following chapters present the isolation and examination of the biological activities of purified constituents from *E. purpurea*.

CHAPTER 2.

CYCLOOXYGENASE AND MOSQUITOCIDAL ALKAMIDES FROM *ECHINACEA PURPUREA* (L.) MOENCH ROOTS*

ABSTRACT

Alkamides from the roots of *Echinacea purpurea* (L.) Moench were examined for anti-inflammatory activity in an in vitro model system. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activities were assessed at pH 7 for alkamides isolated from *E. purpurea* roots to compare inhibitory activities between the two cyclooxygenase isozymes. At 100 μg/mL, several *E. purpurea* alkamides inhibited COX-1 and COX-2 enzymes in the range of 36-60% and 15-46%, respectively, when compared to controls. Mosquitocidal activity was assessed at 100 and 10 μg/mL, with 100% mortality against *Aedes aegyptii* L. larvae noted for several *E. purpurea* alkamides at 100 μg/mL.

* Clifford, L.J.; Nair, M. G.; Rana, J.; Dewitt, D. L. *Phytomedicine*. 9, 249-254, 2002.

INTRODUCTION

Echinacea purpurea is one of three *Echinacea* species widely used for phytoceutical purposes. Initially used by North American Indians to treat various infections, the use of *Echinacea* spread to Europe where *Echinacea*-based products have flourished in the natural product supplement market (Bauer & Wagner, 1991). Following the establishment of *Echinacea* products in the European market, interest in *Echinacea* spp. has increased in the North American nutraceutical market, where the popularity of *Echinacea* products has grown as a complement to modern medicine (Borchers et al., 2000). Validation of the safety and efficacy of herbal supplements would aid in promoting consumer confidence.

Many adverse side effects, including upper gastrointestinal irritation and ulceration, have been reported after long-term use of COX-1 inhibitors such as aspirin (Loll, 1996). COX-1 is the constitutive form of the enzyme responsible for basic regulatory functions in cells and is involved in the production of prostaglandins (Cryer and Dubois, 1998). Since prostaglandins are responsible for such physiological processes including control of gastric secretions and maintenance of mucosal integrity (Ford-Hutchinson, 1996), inhibition of COX-1 might lead to adverse side effects noted in long-term non-steroidal anti-inflammatory drug (NSAID) use. COX-2, the cyclooxygenase isozyme inducible in response to inflammation (Lipsky, 1999), is believed to be a more viable target to inhibit inflammation without the adverse side effects noted with COX-1

inhibitors (Pairet et al., 1996; Vane and Botting, 1996). Compounds with relatively greater COX-2 inhibition compared to COX-1 have demonstrated anti-inflammatory activity with a reduction of adverse side effects when examined in preclinical trials (Ford-Hutchinson, 1996). Specific or greater inhibition of COX-2 as opposed to COX-1 is a desirable target in developing pharmaceutical and phytoceutical products due to the potential reduction in adverse side effects.

Numerous bioactive compounds have been isolated from *Echinacea* spp. Extracts and compounds from various parts of *Echinacea* spp. plants have demonstrated anti-viral, antioxidant, and anti-inflammatory activities (Facino et al., 1995; Bauer & Wagner, 1991; Wagner et al., 1988; Luettig et al., 1989). Of particular interest are the alkamides isolated from *E. purpurea* and *E. angustifolia*, some of which have been examined for in vitro anti-inflammatory activity. Inhibition of 5-lipoxygenase and cyclooxygenase enzymes isolated from ram seminal vesicles by alkamides present in *E. angustifolia* and *E. purpurea* has previously been reported (Müller-Jakic et al., 1994). Two cyclooxygenase isoforms have been identified, COX-1 and COX-2; however, a comparative assessment of the COX-1 versus the COX-2 inhibitory properties of the alkamides has not been examined. In this paper we report the comparative inhibition exhibited by *E. purpurea* alkamides against COX-1 and COX-2.

In addition to anti-inflammatory properties, alkamides from a variety of plants have been reported to possess significant mosquitocidal activity. The mosquitocidal activity of dodeca-2*E*,4*E*,8*E*,10*Z*-tetraenoic acid isobutylamide, an alkamide isolated from *Spilanthes mauritiana* (Jondiko, 1986), is of particular

interest due to its close structural similarity to an alkamide identified in *E. purpurea*. The mosquitocidal activities of the majority of alkamides from *E. purpurea* have not been determined, and are presented here.

MATERIALS AND METHODS

General Experimental

Preparative HPLC was performed on a Model LC-20 Preparative Recycling Liquid Chromatograph (Japan Analytical Industry Co., Ltd., Tokoyo, Japan) with two JAIGEL-C_{18} columns (10 μm, 20 mm x 250 mm) in tandem and UV detection at 260 nm. A gradient of methanol:water, 50-80% methanol over 2 h, 2 mL/min, was used to separate the alkamides into crude fractions. Final purification was achieved using a gradient of acetonitrile:water, with 40-60% acetonitrile over 2 h, 2 mL/min. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Inova 300 MHz spectrometer. Mass spectra were recorded using a Waters Alliance HT LC/MS system (Milford, Massachusetts) with a 2690 Separations Module and Micromass detector, ionization mode AP+ and cone voltage 40eV, with a mobile phase of 40-80% acetonitrile over 30 min. Identification of *E. purpurea* alkamides was made by ^{1}H NMR experiments and by comparison to literature (Bauer et al., 1988) and LC/MS values.

Plant Material

Echinacea purpurea roots were obtained from Trout Lake Farm, (Trout Lake, Washington). Dried, milled *E. purpurea* roots (989.9 g) were exhaustively

extracted with dichloromethane (4 L x 3, 3 days) and concentrated in vacuo. Following concentration, the dichloromethane extract (10.1 g) was separated into methanol-soluble (7.1 g) and insoluble (3.0 g) fractions with the addition of methanol (750 mL x 3). The methanol-soluble portion was concentrated in vacuo, dissolved in chloroform (250 mL), and hexane was added. The supernatant, containing the crude alkamides, was concentrated (4.2 g) and subjected to separation by preparative high-performance liquid chromatography (HPLC).

Cyclooxygenase Inhibitory Assay

An in vitro COX-inhibition model system was used to assess the anti-inflammatory activity of the alkamides isolated from *E. purpurea*. The COX-1 used in the assay was obtained from ram seminal vesicles (Oxford Biomedical Research, Inc., Oxford, MI). COX-1 was obtained from microsomal preparations of ram seminal vesicles according to previously reported methods (Laneuville et al., 1994; Meade et al., 1993). A recombinant human microsomal preparation of COX-2 was provided by Dr. David Dewitt (Michigan State University, East Lansing, MI). The assay was conducted in a 600 μL Instech chamber (Instech Laboratory, Plymouth Meeting, PA) maintained at 37 °C, containing a reaction buffer of 0.1 M Tris, 1 mM phenol, 100 μM arachidonic acid, and 17 μg hemoglobin. Alkamides were dissolved in DMSO such that a 10 μL aliquot would yield a final assay concentration of 100 μg/mL. The reaction was initiated with either a 10 μL aliquot of COX-1 or a 20 μL aliquot of COX-2. Oxygen uptake was

monitored using a YSI model 5300 biological oxygen monitor (Yellow Springs Instruments, Inc., Yellow Springs, OH) and recorded using Quicklog for Windows, version 1.0 (Strawberry Tree, Inc., Sunnyvale, CA).

Aspirin, ibuprofen, naproxen, celecoxib (Celebrex™), and rofecoxib (Vioxx™) were dissolved in DMSO and used as controls for both the COX-1 and COX-2 inhibitory assays. Aspirin was tested at 1000 μM (180 μg/mL), ibuprofen at 10 μM (2.06 μg/mL), and naproxen at 10 μM (2.52 μg/mL) for both COX-1 and COX-2. Celecoxib (Celebrex™) and rofecoxib (Vioxx™) were obtained as physican's professional samples (Dr. Subash Gupta, Sparrow Pain Center, Sparrow Hospitals, Michigan), ground to fine powder, dissolved in DMSO, and tested at 1.6 μg/mL for both COX-1 and COX-2.

Mosquitocidal Assay

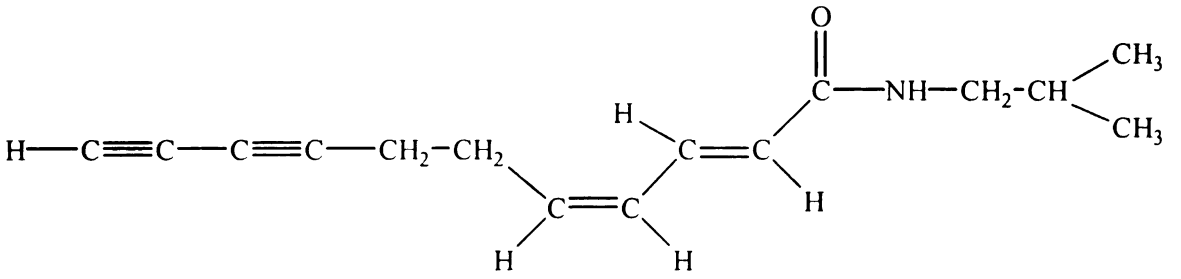
The mosquitocidal assay was performed according to previously published methods (Kelm et al., 1998; Nair et al., 1989). *Aedes aegyptii* L., provided by Dr. Alan Hayes (Department of Entomology, Michigan State University) were hatched and raised in 500 mL of degassed distilled water, with approximately 5 mg of bovine liver powder added for nourishment. After the mosquito larvae had reached the fourth-instar (four days), the larvae were prepared for the bioassay. Ten to fifteen larvae in 980 μL of degassed, distilled water were placed in four mL culture tubes. Stock solutions of the alkamides in DMSO were prepared such that a 20 μL aliquot added to the mosquito larvae in the bioassay culture tubes yielded a final assay concentration of 100 μg/mL or 10 μg/mL. Alkamide assay

concentrations in μM are noted in **Appendix A**. A 20 μL aliquot of DMSO was used as the control. The alkamides were tested at 100 μg/mL and 10 μg/mL and were tested in triplicate with the control set. Dead larvae were recorded at time zero, 1, 2, 4, 9, and 24 h and reported in terms of percent mortality.

RESULTS

The bioactive alkamides isolated from the dried *E. purpurea* roots (**Figure 2.1**) were identified as undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**1**), undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide (**2**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**3**), undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**4**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**5**), and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*Z*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**6/7**). Compounds 6 and 7 were inseparable under various HPLC conditions. Yields obtained were 8 mg (**1**), 14 mg (**2**), 21 mg (**3**), 12 mg (**4**), 15 mg (**5**), and 40 mg (**6/7**).

The results of the mosquitocidal assay are summarized in **Figure 2.2**. The mixture of alkamides **6/7** proved to be the most effective in the mosquitocidal assay, with 87.5% mortality of mosquito larvae within fifteen minutes when assayed at a concentration of 100 μg/mL. Lowering the concentration to 10 μg/mL still yielded significant mosquitocidal activity, with 63% mortality in one hour (**Figure 2.2**). Mosquito larvae that were still regarded as alive showed significant impairment at one hour and beyond for alkamides **6/7**. Compound **1**



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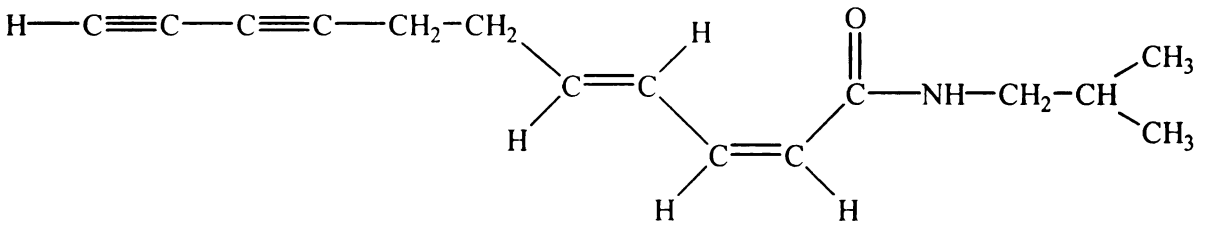

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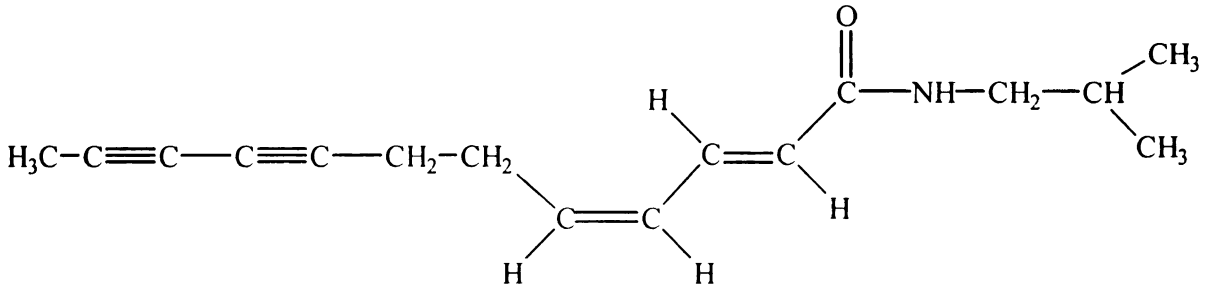

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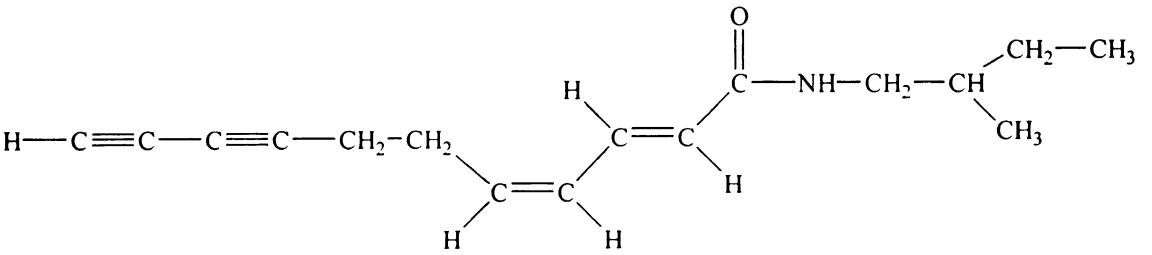
Figure 2.1. Alkamides isolated from *E. purpurea* roots: undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**1**), undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide (**2**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**3**), undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**4**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**5**), and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**6/7**).



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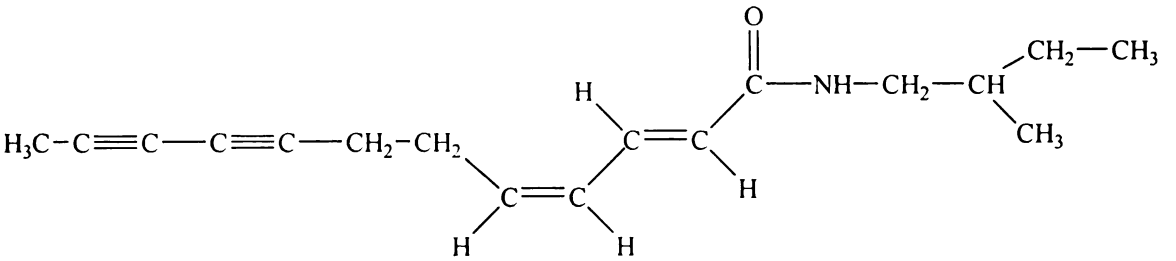

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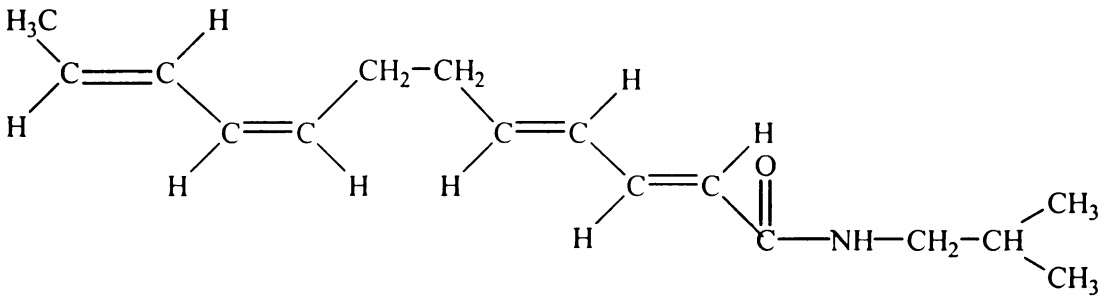
Figure 2.1 (cont'd). Alkamides isolated from *E. purpurea* roots: undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**1**), undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide (**2**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**3**), undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**4**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**5**), and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**6/7**).



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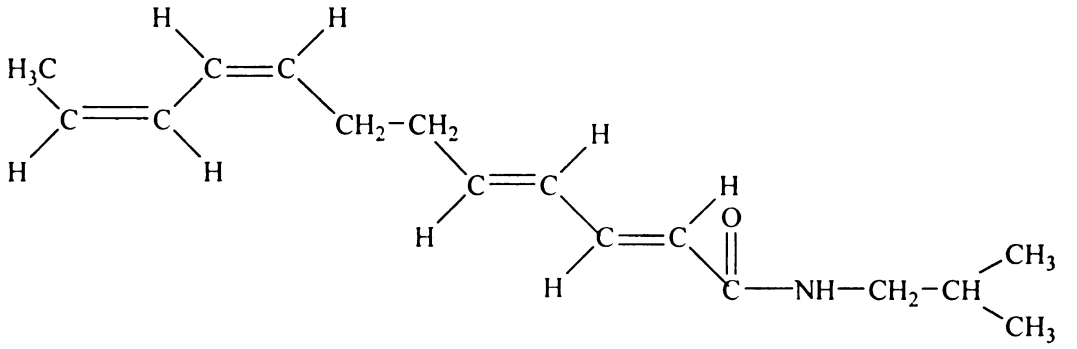

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Figure 2.1 (cont'd). Alkamides isolated from *E. purpurea* roots: undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**1**), undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide (**2**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**3**), undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**4**), dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**5**), and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**6/7**).

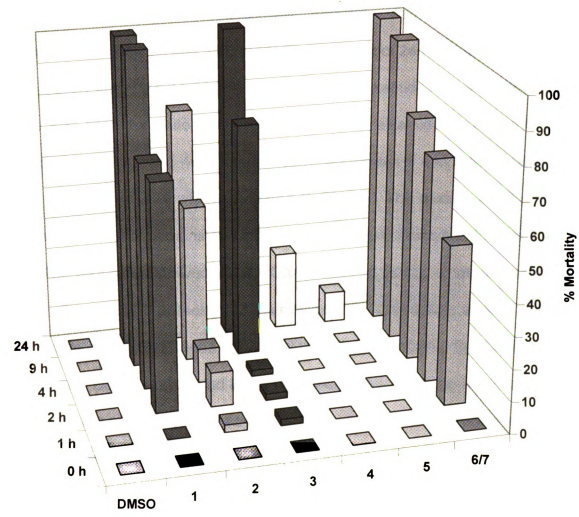


Figure 2.2. Mosquitocidal activity of *E. purpurea* alkamides. Alkamides **1-5** were assayed at 100 μg/mL and the mixture of **6/7** was assayed at 10 μg/mL. DMSO was used as the solvent control.

demonstrated slightly less activity with 71 and 100% mortality by 2 and 9 h, respectively. Compounds **2** and **3** showed mosquitocidal activity at the end of 9 h, with 78 and 50% mortality, respectively. Alkamides **4** and **5** proved to be the least active, with only 25 and 10% mortality at the end of 24 h, respectively.

COX-1 and COX-2 inhibitory activities of the alkamides are summarized in **Figure 2.3**. COX-1 inhibitory activity was highest for alkamides **2**, **4**, and **5**, with inhibitions of 60, 55, and 48%, respectively. Alkamides **1** and **3** both exhibited COX-1 inhibition of 36% when compared to DMSO. The mixture of alkamides **6/7**, however, did not demonstrate inhibition of the COX-1 enzyme (**Figure 2.3**).

The alkamides showed lower COX-2 inhibitory activity when compared to COX-1. Compound **2** possessed the strongest COX-2 inhibitory activity at 46%. Compounds **4** and **5** showed 39 and 31% inhibition of the COX-2 enzyme, respectively. Compounds **1** and **3** had the lowest activities of the alkamides, with each exhibiting 15% inhibition. As with COX-1, the mixture of alkamides **6/7** did not demonstrate activity against COX-2.

Discussion

Numerous plant species in the Asteraceae possess insecticidal alkamides (Greger, 1984), and the mosquitocidal properties of *E. angustifolia* have previously been reported (Hartzell, 1947; Greger, 1984). Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide, an alkamide isolated from *S. mauritiana* (Jondiko, 1986) similar in structure to **6/7** identified in *E. purpurea* (Bauer et al., 1988) and

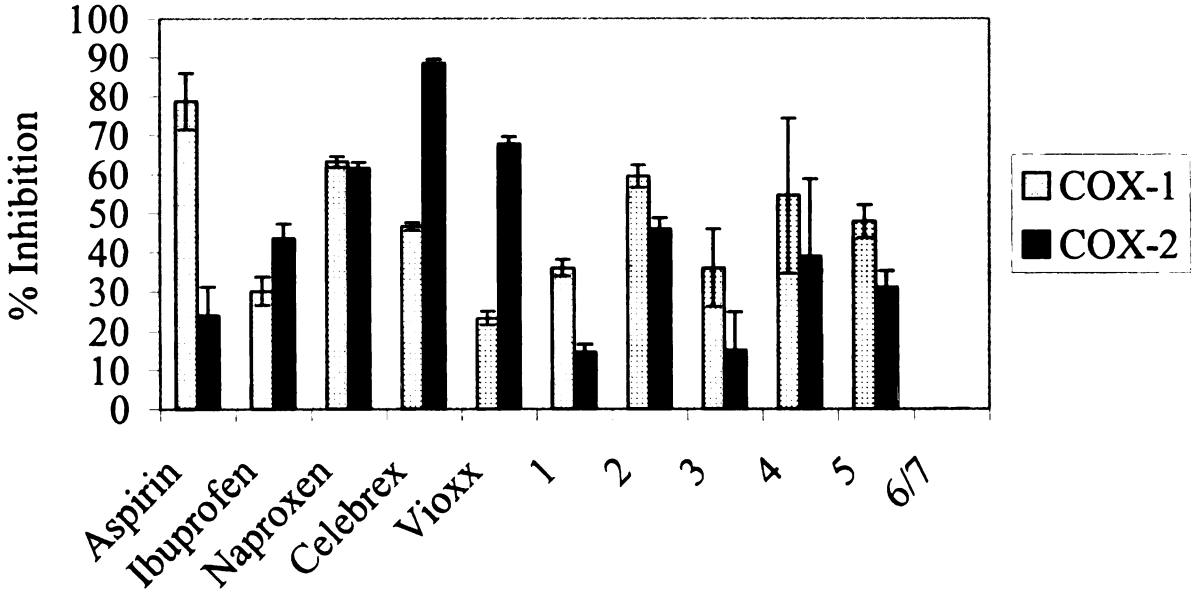


Figure 2.3. Comparative inhibition of COX-1 and COX-2 enzymes by *E. purpurea* alkamides. Alkamides were assayed at 100 μg/mL and compared to the solvent control, DMSO. Aspirin, ibuprofen, naproxen, celecoxib (Celebrex™), and rofecoxib (Vioxx™) were used as controls. Aspirin was tested at 1000 μM (180 μg/mL), ibuprofen at 10 μM (2.06 μg/mL), and naproxen at 10 μM (2.52 μg/mL) for both COX-1 and COX-2. Celecoxib (Celebrex™) and rofecoxib (Vioxx™) were tested at 1.6 μg/mL for both COX-1 and COX-2. Vertical bars represent the standard deviation of each data point (n=2).

E. angustifolia (Bauer et al., 1991), showed 100% mortality at 24 h against *A. aegypti* at 10^{-5} mg/mL (Jondiko, 1986). In our study, the high activity noted with alkamides **6** and **7** from *E. purpurea* supports the mosquitocidal activity indicated from *E. angustifolia*.

Interestingly, both 2-methylbutylamides, **4** and **5**, demonstrated the lowest mosquitocidal activity in the assay when compared to the isobutylamides tested. The presence of an *E/Z* configuration of the double bonds is regarded as important for conveying the highest degree of insecticidal and thus biological activity (Greger, 1984). From the results of the mosquitocidal assay, it appears that the 2-methyl versus an isobutyl functionality might also play a role in the effectiveness of alkamides as mosquitocidal compounds.

Previously, eight alkamides from *E. angustifolia* D.C. and ten alkamides from various *Achillea* species were examined for cyclooxygenase and 5-lipoxygenase activities (Müller-Jakic et al., 1994). A mixture of **6/7**, isolated from *E. angustifolia* roots, demonstrated inhibition of 54.7% of COX from microsomal sheep seminal vesicle preparations at a concentration of 50μg/ml. The mixture of **6/7** isolated from *E. purpurea* roots in our study did not show activity at 100 μg/mL for either COX-1 or COX-2. Alkamides **1-5** from *E. purpurea* roots have not been examined for cyclooxygenase inhibitory activity; however, undeca-2*E*,4*E*-diene-8,10-diynoic acid isobutylamide, isolated from *Achillea millefolium* L., was shown to inhibit COX-1 enzyme at 40% (Müller-Jakic et al., 1994) while compounds **1** and **2** exhibited 36 and 60% COX-1 enzyme inhibition, respectively.

Compound **2** exhibited the highest inhibition against both COX-1 and COX-2 enzymes. The 2-methylbutylamides, compounds **4** and **5**, demonstrated higher COX-1 and COX-2 inhibitory activity when compared to the majority of the isobutylamides tested, with the exception of compound **2**. Compounds **1** and **3**, differing in one methyl group, demonstrated equal inhibitory activities for both COX-1 and COX-2 enzymes, indicating that the inhibition of COX-1 and COX-2 enzymes is unaffected by the addition of a single methyl group.

The *E. purpurea* alkamides examined in this study did not show selective inhibition of COX-2 enzyme. Additional work in identifying the structure activity relationships of *E. purpurea* alkamides and alkamides of similar structure differing in *E/Z* configurations would aid in determining the potency of these alkamides for specific phytoceutical applications.

CHAPTER 3.

THE ISOLATION AND PURIFICATION OF BIOACTIVE COMPOUNDS FROM *ECHINACEA PURPUREA* (L.) MOENCH FLOWERS

ABSTRACT

Echinacea purpurea (L.) Moench is one of three *Echinacea* species commonly used for phytoceutical purposes. While the biological activity of *Echinacea angustifolia, Echinacea pallida*, and *E. purpurea* roots have been examined, the flowers have not been studied to the same extent. In order to identify bioactive constituents from this underutilized part of *E. purpurea*, several compounds were isolated from lyophilized *E. purpurea* flowers to later determine their activity through various in vitro studies. Three flavonoids, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside, were purified from methanolic extracts of lyophilized *E. purpurea* flowers. In addition, two anthocyanins, cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside, were obtained from a methanol extract of lyophilized *E. purpurea* flowers. The isolation, purification, and structure elucidation of these compounds are presented.

INTRODUCTION

Echinacea purpurea is widely used as a natural product supplement. Initially used by North American Indians to treat various infections, the popularity of *Echinacea* spread to Europe, where *Echinacea* products have flourished in the nutraceutical market (Bauer & Wagner, 1991). Interest in *Echinacea* spp. has recently increased in the North American phytoceutical market, and *Echinacea* products are currently used as a complement to modern medicine (Borchers et al., 2000). A resurgence in the popularity of *Echinacea* in the United States during the late twentieth century has made it one of the top-selling herbal supplements (Gunning, 1999).

Traditionally, *Echinacea* spp. were used both externally and internally to treat ailments including burns, insect bites, wounds, aches, chills, rheumatism, arthritis, mumps, and measles (Bauer and Wagner, 1991; Kindscher, 1989). *Echinacea* roots were used most often; however, juice from whole *Echinacea* plants and infusions brewed from the fresh herb were occasionally consumed as a medicinal remedy. Currently, *Echinacea* preparations are sold in the form of tinctures, tablets, capsules, juices, and teas from the roots or fresh herb of the plant (Gunning, 1999; Li and Wang, 1998). Extracts prepared solely from the flowers of *Echinacea* are not currently commercially available in the United States.

Numerous bioactive compounds have been isolated from *Echinacea* spp. Caffeic acid derivatives, flavonoids, alkaloids, alkamides, polyacetylenes, and

anthocyanins have been obtained from various parts of *Echinacea* spp. plants, with the majority isolated from the roots and leaves (Bauer and Wagner, 1991). Occasionally, the whole *Echinacea* plant, referred to as the fresh herb, is used to prepare extracts for biological testing or isolation; however, the flower is included only if the plant is in bloom. Very few studies have examined the bioactive compounds present in the flowers of *E. purpurea* (Cheminat et al., 1989). The vast majority of the research performed has been on the roots, stems, and leaves of *Echinacea* (Bauer and Wagner, 1991).

Extracts and compounds from various portions of *Echinacea* plants have demonstrated anti-viral, antioxidant, and anti-inflammatory activities (Facino et al., 1995; Bauer & Wagner, 1991; Wagner et al., 1988; Luettig et al., 1989). *Echinacea* root and herb extracts have been tested in clinical trials to determine the effects on relieving the severity or duration of upper respiratory tract infections (Gunning, 1999; Brinkeborn et al., 1998; Hoheisel et al., 1997; Bräunig et al., 1992). The anti-viral, antioxidant, and anti-inflammatory properties of extracts and compounds isolated from the flowers of *E. purpurea* are unknown. Identification of the bioactive constituents from *E. purpurea* flowers would provide useful information with regard to herbal supplement preparation.

The purpose of this research was to isolate and identify constituents from *E. purpurea* flowers for subsequent analysis to determine the bioactive properties of compounds from *E. purpurea* flowers. The extraction, isolation, and structure elucidation of three flavonoids and two anthocyanins from *E. purpurea* flowers are presented here.

MATERIALS AND METHODS

General Experimental

Amberlite XAD-16 resin was obtained from Supelco (Bellefonte, PA). LC-SORB SP-A-ODS gel (particle size 25-40 μm) was purchased from Dychrom (Santa Clara, CA). Silica gel PTLC plates (20 x 20 cm, 1000 μm) were obtained from Analytech Inc. (Newark, DE). Preparative HPLC for flavonoids was performed on a Model LC-20 Preparative Recycling Liquid Chromatograph (Japan Analytical Industry Co., Ltd., Tokoyo, Japan) with a JAIGEL-C_{18} column (10 μm, 20 mm x 250 mm) and UV detection at 210 nm. Analytical HPLC analysis of the anthocyanin fractions was performed using Millennium 2010 Chromatography Manager version 3.05.01 (Waters Corp., Milford, MA) with Capcellpak C_{18} column (4.6 x 250 mm, 5 μm) (Dychrom, Sunnywale, CA) and a mobile phase of acetonitrile: H_2O (85:15) with 4% H_3PO_4 in the aqueous phase. Anthocyanins were detected at λ 520 nm using a Waters PDA detector (Waters Corp., Milford, MA). Fractions containing anthocyanins were concentrated in vacuo and passed over XAD-16 to remove the phosphoric acid. Fractions were applied to the XAD-16 column, washed with H_2O until a neutral pH was obtained, and eluted with 100% MeOH. Following elution, each fraction from the XAD column was reduced in vacuo and the anthocyanins were stabilized with several drops of MeOH spiked with HCl, yielding a pH of approximately 2.

HPLC-ES/MS of anthocyanins were recorded using a MicroMass Quattro II LC-MS/MS system (Micromass, Waters Corp., Beverly, MA) equipped with a

Waters 2090 HPLC pump and Waters 996 PDA detector (Waters Corp., Milford, MA) with an HP ODS Hypersil HPLC column (4.0 x 125 mm, 5 μm) (Agilent Technologies, Wilmington, DE). Data handling was performed using MassLynx version 3.4 software (Micromass, Waters Corp., Beverly, MA). MS parameters were set to ionization mode AP+ and cone voltage 20 eV. Conditions of analysis were as follows: injection volume 10 μL; column temperature 30 °C; PDA range 200-799 nm, with 520 nm as the detection wavelength. Mobile phase conditions were as follows: Solvent A) 0.1% TFA/H_2O (v/v), B) 50.4% H_2O/48.5% ACN/1.0% CH_3COOH/0.1% TFA (v/v/v/v); gradient % B: initial: 20%, 26 min: 60%, 30 min: 20%, 35 min: 20%; run time 35 min; flow rate 0.80 mL/min. ^1H and ^{13}C NMR spectra of the flavonoids and anthocyanins were recorded in DMSO-d_6 and CD_3OD with 0.1 mL of DCl, respectively, on a Varian Inova 300 MHz spectrometer (Palo Alto, CA). The structures of the flavonoids were determined and compared to the ^1H NMR and ^{13}C data of published literature values (Markham et al., 1982). Anthocyanins were identified based on comparison of ^1H NMR and ^{13}C data to literature values (Fossen and Andersen, 1998; Agrawal and Mahesh, 1989), and by MS and comparison to standard cyanidin-3-*O*-β-D glucopyranoside retention times using analytical HPLC.

Plant Material and Extraction

Echinacea purpurea (L.) Moench plants were obtained from Dr. Erik Runkle (Michigan State University, East Lansing, MI) and maintained in the Michigan State University Bioactive Natural Product and Phytoceuticals Laboratory greenhouses. *Echinacea purpurea* flowers were harvested over a

period of eight months from approximately fifty, two-year-old plants. Extraction of *E. purpurea* flowers was carried out through the scheme in **Figure 3.1**. Fresh *E. purpurea* flowers (827 g) were lyophilized, ground, and extracted exhaustively with MeOH (4 L x 3). The crude MeOH extract (193 g) was concentrated in vacuo, dissolved in MeOH-H_2O (85:15), and partitioned with hexane (1 L x 3). The MeOH- H_2O soluble portion (137 g) was subjected to XAD-16 resin column chromatography. The column was sequentially eluted with H_2O (4 L), 50% MeOH (3 L), and 100% MeOH (3 L). The 50% MeOH eluate afforded 23 g of rust-colored gum from which the anthocyanins were isolated. The 100% MeOH eluate yielded 10 g of brown extract, from which the flavonoids were obtained.

Purification of anthocyanins

Purification of the anthocyanins from the 50% MeOH XAD eluate (23 g) was achieved by repeated gradient MPLC with 10-50% acetonitrile (4% H_3PO_4 in the aqueous phase). Ten grams of the 50% MeOH XAD eluate were applied to a C_{18} MPLC column, and eluted with acetonitrile: H_2O (10:90 to 50:50) with 4% H_3PO_4 in the aqueous phase under gradient conditions. Ten fractions (fractions A1-A10) of 200-450 mL were collected and analyzed for anthocyanins by analytical HPLC.

Anthocyanin-containing fractions A7 and A8 were combined (1.57 g) and subjected to C_{18} MPLC with acetonitrile: H_2O (10:90 to 20:80) with 4% H_3PO_4 in the aqueous phase under gradient conditions. One hundred twenty, 12 mL fractions were collected, and every fifth fraction was screened for anthocyanins by analytical HPLC. Fractions containing anthocyanins were combined,

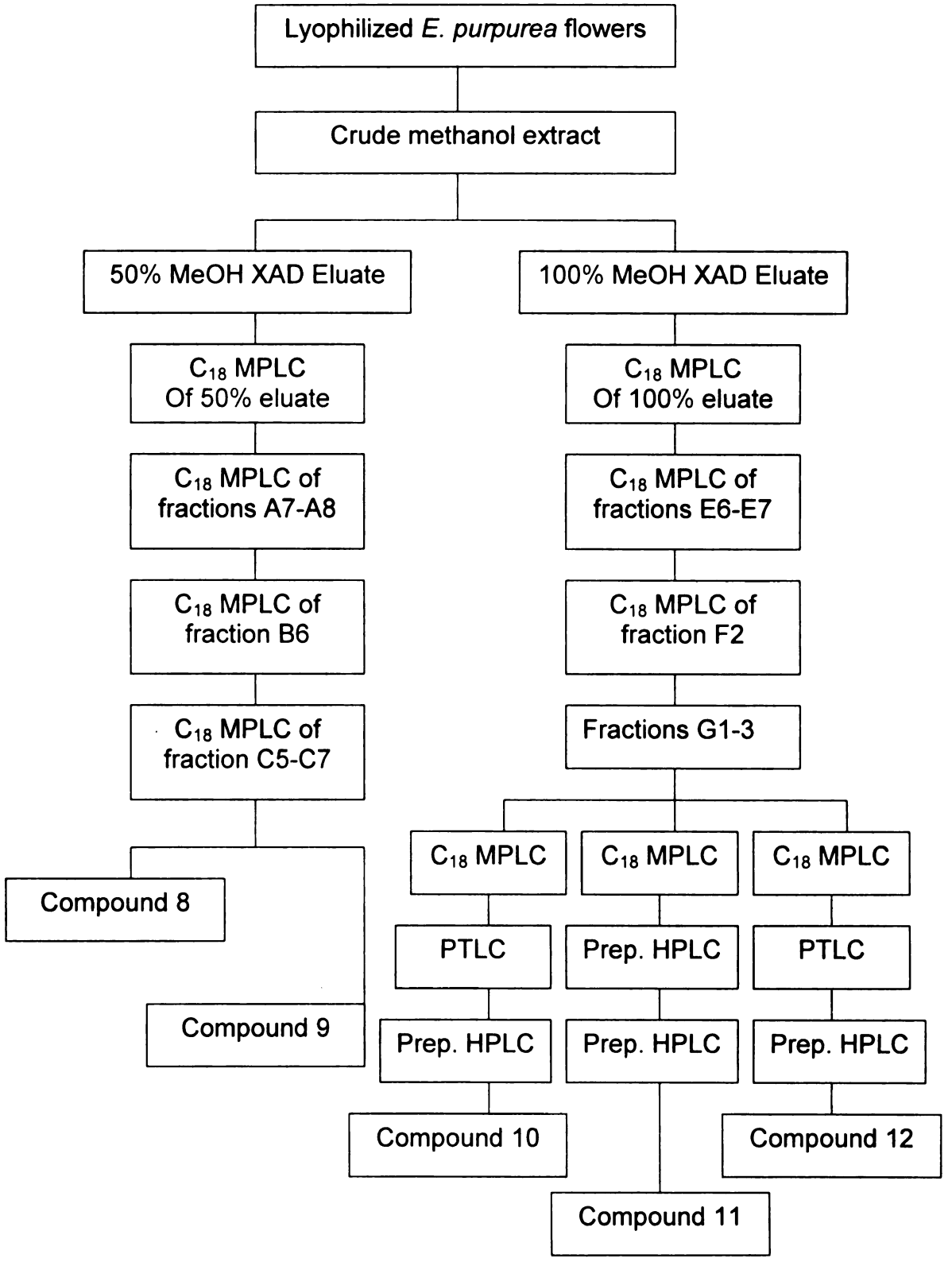


Figure 3.1. Extraction scheme for anthocyanins and flavonoids isolated from *E. purpurea* flowers.

concentrated, and passed over XAD to remove the phosphoric acid, yielding seven fractions (B1-B7).

Fraction B6 was further subjected to C_{18} MPLC under the previously described conditions. Eighty, 12 mL fractions were collected and analyzed by analytical HPLC. Fractions with similar profiles were pooled yielding eight fractions (C1-C8). Further C_{18} MPLC of fractions C5-C7 (135 mg) was carried out with acetonitrile: H_2O (10:90 to 15:85), with 4% H_3PO_4 in the aqueous phase, under gradient conditions. Ninety, 12 mL fractions were collected and analyzed by analytical HPLC, yielding eight fractions (D1-D8). Compounds **8** (13 mg) and **9** (8 mg) were obtained as red, amorphous solids.

Purification of flavonoids

The 100% MeOH XAD eluate (10 g) was subjected to C_{18} MPLC with a gradient of 40-100% MeOH with 0.02% TFA. Ten, 200 mL fractions were collected (E1-E10) and concentrated in vacuo. Fractions E6 and E7 were combined (5.6 g) and subjected to further C_{18} MPLC with 40-70% MeOH. One hundred, 12 mL fractions were collected and every fifth fraction was analyzed by TLC. Fractions with similar flavonoid profiles were combined, yielding fractions F1-F3. MPLC on fraction F2 (1.79 g) was performed with a mobile phase of 10-50% acetonitrile, which yielded eighty, 12 mL fractions. Fractions were analyzed by TLC and combined, yielding fractions G1-G4.

Fraction G1 (600 mg) was separated by C_{18} MPLC with a mobile phase of 20-75% acetonitrile. Sixty, 12 mL fractions were collected, analyzed by TLC, and combined into six fractions (H1-H6). Fraction H1 (173 mg) was subjected to

PTLC with dichloromethane:MeOH:toluene:formic acid (12:4:0.5:0.5), yielding two bands. Band I2 (23 mg) was further purified by repeated C_{18} preparative HPLC with a mobile phase of 30% acetonitrile, yielding compound **10** (12 mg) as yellow crystals when recrystallized from methanol.

Fraction G2 (538 mg) was subjected to further C_{18} MPLC using a mobile phase gradient of 10-40% acetonitrile. Fifty, 12 mL fractions were collected, concentrated, and combined yielding four fractions, J1-J4, based on TLC profiles. Fraction J2 (235 mg) was further purified by repeated C_{18} preparative HPLC with a mobile phase of 20% acetonitrile. Two fractions were collected (K1-K2), and fraction K2 (29 mg) was further purified by repeated preparative HPLC with a mobile phase of 30% acetonitrile, yielding compound **11** (15 mg) as yellow crystals after crystallization from MeOH.

Fraction G3 (600 mg) was subjected to C_{18} MPLC with 20-50% acetonitrile under gradient conditions. One hundred twenty, 12 mL fractions were collected, analyzed by TLC, and combined into six fractions (L1-L6). Silica PTLC on fraction L2 (56 mg) with dichloromethane:MeOH:toluene:formic acid (12:4:0.5:0.5) yielded three bands (M1-M3). Purification of fraction M2 (7 mg) by repeated preparative HPLC with a mobile phase of 30% acetonitrile yielded compound **12** (2.6 mg) as a yellow, amorphous solid.

RESULTS AND DISCUSSION

Purification of anthocyanins

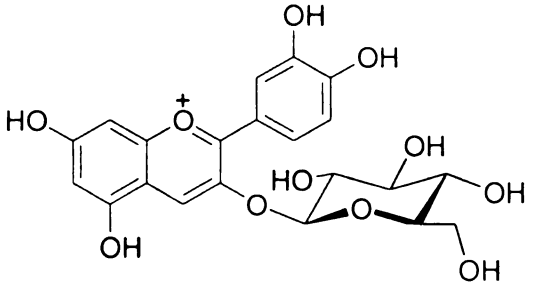
Compound **8** was identified as cyanidin-3-*O*-β-D glucopyranoside (**8**) based on ^{1}H and ^{13}C chemical shifts (Fossen and Andersen, 1998) (**Table 3.1**), and by comparative analytical HPLC and HPLC/MS values. Compound **9** was identified as cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) based on ^{1}H and ^{13}C chemical shifts (Fossen and Andersen, 1998) (**Table 3.1**) and by HPLC/MS values. The structures of compounds **8** and **9** are shown in **Figure 3.2**.

The overwhelming presence of compound **8** in the crude MeOH extract from XAD and initial MPLC fractions A1-A10 suggested that compound **8** was the predominant anthocyanin present in *E. purpurea* flowers. Hydrolysis of the 50% MeOH extract with concentrated HCl for 16 h at room temperature yielded only the one aglycon, cyanidin, based on analytical HPLC analysis and comparison to a cyanidin standard. Cyanidin has been reported to be the predominant aglycon in purple flowers (Saito and Harborne, 1992), and anthocyanin **9** has been reported in a variety of blue and purple flowers (Akashi et al., 1997; Saito and Harborne, 1992; Kim et al., 1989; Bridle et al., 1984). The deep purple-red color of compound **9** likely contributes significantly to the pink-purple hue of *E. purpurea* petals.

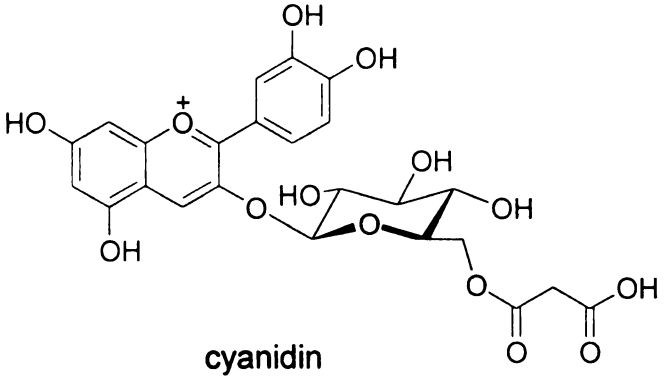
While only **8** and **9** were obtained pure, additional anthocyanins were present in anthocyanin-enriched MPLC fractions C1-C4 and in B1-B3. Isolation

Table 3.1. ^{13}C Chemical shifts for anthocyanins from *E. purpurea*.

	Compounds	
Carbon No.	**8**	**9**
2	164.3	164.5
3	145.6	145.7
4	137.0	167.1
5	159.1	159.0
6	103.4	103.4
7	170.4	170.1
8	95.2	95.2
9	157.7	157.5
10	113.4	115.4
1'	121.3	121.3
2'	118.4	118.9
3'	147.3	147.4
4'	155.7	155.7
5'	117.4	115.5
6'	128.3	130.9
1''	103.7	103.6
2''	74.8	74.8
3''	78.0	78.7
4''	71.1	71.1
5''	78.7	75.3
6''	62.3	65.1
6'' malonyl		
1'''		167.9
2'''		41.6
3'''		169.2



cyanidin
3-O-β-D-glucopyranoside



cyanidin
3-O-malonyl-(1→6)-β-D-
glucopyranoside

Figure 3.2. Anthocyanins isolated from *E. purpurea* flowers: cyanidin-3-*O*-*β*-D-glucopyranoside (**8**) and cyanidin-3-O-malonyl-(1→6) -*β*-D-glucopyranoside (**9**).

of these extremely polar compounds, present in very low amounts, was not successful by MPLC or preparative HPLC. LCMS failed to identify the mass of these anthocyanins due to insufficient quantities. Previous attempts to isolate anthocyanins from *E. purpurea* and *E. pallida* resulted in the isolation of compounds **8** and **9** in addition to two unidentified acylated anthocyanins (Cheminat et al, 1989). Acylation has been reported to increase retention times by two-fold when compared to non-acylated anthocyanins (Kim et al., 1989). During analytical HPLC analysis, compound **9** eluted significantly later than compound **8**. Based on the HPLC retention time of compound **9** relative to compound **8**, it is unlikely that the unidentified anthocyanins in fractions C1-C4 and B1-B3, which eluted prior to compound **8**, were the same acylated anthocyanins previously suggested in *E. purpurea* (Cheminat et al., 1989).

There are conflicting reports regarding the stability of anthocyanin **9**. Recently, it was reported that compound **9** was stable (Luczkiewcz and Cisowski, 2001); however, the work performed here confirms earlier reports indicating that compound **9** is highly unstable (Kim et al., 1989). Anthocyanins are generally stabilized by the addition of acid to prevent the break-down of the flavylium cation to the colorless quinoidal base. The persistent presence of compound **8** in fractions initially containing only anthocyanin **9** confirmed previous findings of the unstable nature of compound **9**. Loss of the malonyl group occurred rapidly both upon storage and during purification by MPLC. The stability of compound **9** in phytoceutical preparations of *E. purpurea* flowers should be further examined if compound **9** is a desired component of the product.

Purification of flavonoids

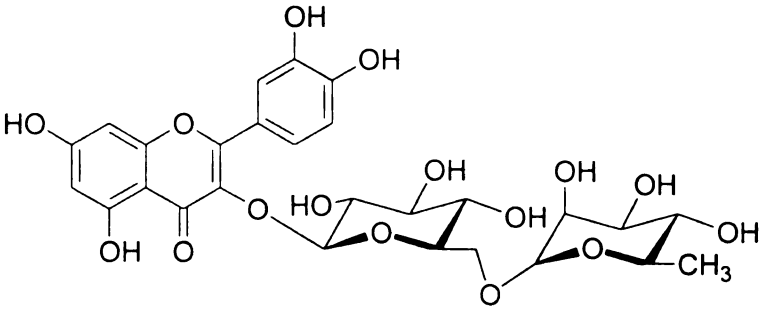
Compounds **10-12** were identified based on ^{1}H and ^{13}C chemical shifts (**Table 3.2**) and in agreement with published data (Markham et al. 1982) as quercetin-3-*O*-rutinoside (**10**), kaempferol-3-*O*-robinobioside (**11**), and quercetin-3-*O*-glucoside (**12**). The structures of these flavonoids are presented in **Figure 3.3**.

Numerous flavonoid-type compounds were noted in the MPLC column fractions (G1-G3) and preparative HPLC isolation (I1, K1, and M3) of compounds **10-12**. Attempts to isolate the minor flavonoids yielded insufficient masses that were not sufficient to elucidate the structure of these compounds. While more flavonoids are likely to be present in *E. purpurea* flowers, greater quantities of starting material would be required to isolate significant quantities of these compounds in order to elucidate the structures. Based upon the small quantities of compounds **10-12** isolated from the flowers and the minute quantities of unidentified flavonoid-type compounds, it may be safe to suggest that flavonoids appear to be relatively minor constituents of the methanol extract of *E. purpurea* flowers.

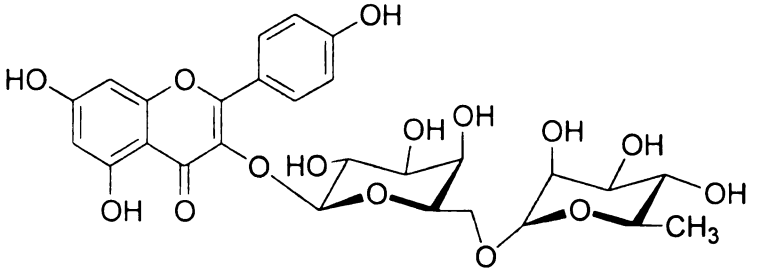
While there have not been any reports regarding the flavonoid content of *E. purpurea* flowers, numerous flavonoids have been reported from the leaves of *E. angustifolia* and *E. purpurea* (Figure 1.5). Quercetin-3-*O*-glucoside has been isolated from *E. purpurea* leaves; however, quercetin-3-*O*-rutinoside and kaempferol-3-*O*-robinobioside have not been reported in *E. purpurea* or *E. angustifolia* leaves (Bauer and Wagner, 1991). The flavonoid content of *E.*

Table 3.2. ^{13}C Chemical shifts for flavonoids isolated from *E. purpurea*.

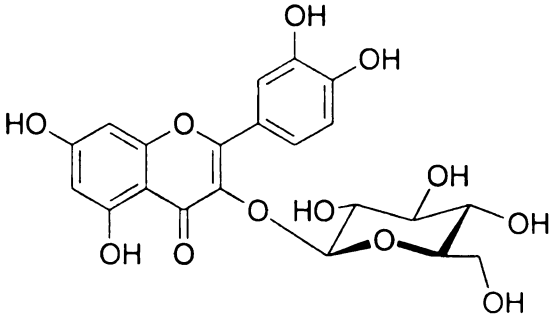
	Compounds		
Carbon No.	**10**	**11**	**12**
2	156.5	156.4	156.3
3	133.3	133.3	133.3
4	177.2	177.3	177.4
5	161.2	161.1	161.2
6	98.9	99.0	98.6
7	164.8	165.2	164.2
8	93.7	93.9	93.5
9	156.5	156.5	156.1
10	103.7	103.5	103.9
1'	121.6	120.8	121.6
2'	115.2	130.9	115.2
3'	144.8	115.4	144.8
4'	148.5	160.0	148.4
5'	116.2	115.1	116.2
6'	121.1	130.9	121.1
1''	101.3	103.5	100.8
2''	74.1	71.9	74.1
3''	76.5	73.0	77.5
4''	70.6	68.0	69.9
5''	68.3	73.5	76.5
6''	67.0	65.3	60.9
1'''	100.8	100.0	
2'''	70.4	70.6	
3'''	70.0	70.4	
4'''	71.9	71.9	
5'''	68.3	68.0	
6'''	17.5	17.9	



quercetin
3-O-α-L-rhamnosyl-(1→6)-
β-D-glucopyranoside



kaempferol
3-O-α-L-rhamnosyl-(1→6)-
β-D-galactopyranoside



quercetin
3-O-β-D-glucopyranoside

Figure 3.3. Flavonoids isolated from *E. purpurea* flowers: kaempferol-3-*O*-robinobioside (**10**), quercetin-3-*O*-rutinoside (**11**), and quercetin-3-*O*-glucoside (**12**).

purpurea leaves has been reported as 0.48% (Bauer and Wagner, 1991). *Echinacea purpurea* flowers appear to contain significantly less flavonoids than the leaves.

Echinacea. purpurea flowers contain several compounds that have been reported to be biologically active. Three flavonoids, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside, and two anthocyanins, cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside, were purified from methanolic extracts of lyophilized *E. purpurea* flowers. *Echinacea purpurea* flower herbal supplement preparations are a potential new source of the bioactive compounds isolated in this study. Examination of *E. purpurea* compounds through various bioassays will lend credence to the phytoceutical effectiveness of *E. purpurea* natural product supplements. Anthocyanins are noted to have high therepeutic value (Luczkiewcz and Cisowski, 2001; Wang et al., 1999), and flavonoids are well established as antioxidants (Pietta, 1999; Rice-Evans, 1999; Arora et al., 1998). Further work to determine the antioxidant and anti-inflammatory activities and the effects on the induction of cytokines IL-6 and TNF-α will be performed using these compounds isolated from *E. purpurea* flowers.

CHAPTER 4.

CYCLOOXYGENASE-1 AND –2 INHIBITORY AND ANTIOXIDANT ACTIVITIES OF COMPOUNDS FROM *ECHINACEA PURPUREA* (L.) MOENCH

ABSTRACT

The popularity of *Echinacea purpurea* (L.) Moench as a natural product supplement has increased in recent years, and products containing *E. purpurea* have grown in both number and variety. Teas and extracts from the aerial portions of the plant, including stems, leaves, and flowers have become increasingly popular; however, little is known about the bioactivity of compounds from *E. purpurea* flowers. Flavonoids (quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside) and anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside), isolated from methanolic extracts of lyophilized *E. purpurea* flowers, were examined for antioxidant and cyclooxygenase–1 (COX-1) and cyclooxygenase–2 (COX-2) inhibitory activities. Caffeic acid, caftaric acid, chlorogenic acid, and cichoric acid, reported components of *E. purpurea*, were also tested. The anthocyanins demonstrated the greatest cyclooxygenase inhibitory activities at 100 μg/mL, with inhibition of COX-1 and COX-2 ranging from 26-28% and 23-26%, respectively. When assayed at 10 μg/mL, the antioxidant activity of these compounds ranged from 33-81% for the anthocyanins, 3-54% for the flavonoids, and 35-79% for the caffeoyl derivatives. The results of these studies indicate that the development of *E. purpurea* flower-based phytoceuticals is warranted.

INTRODUCTION

Echinacea purpurea (L.) Moench is one of three *Echinacea* spp. currently of phytoceutical interest. The popularity of *Echinacea* can be linked to the general increase in the use of herbal supplements to improve quality of life and as a means of disease prevention or management (Ness et al., 1999). Nutraceuticals do not require testing for safety or efficacy prior to marketing (Percival, 2000; Zink and Chaffin, 1998), and as a result, the active components in many commercially available products have not been identified (Mazza and Cottrell, 1999; Melchart et al., 1995). The recent increase in the popularity of *Echinacea* has generated a great deal of research interest to determine the efficacy of this herbal supplement.

Historically, *Echinacea* roots were used to alleviate pain and inflammation (Bauer and Wagner, 1991); however, teas prepared from the fresh herb were also consumed to treat conditions such as rheumatism, arthritis, mumps, and measles (Kindscher, 1989). Currently, *Echinacea* extracts are available in a variety of forms, such as tablets, capsules, tinctures (alcoholic extracts), juices, and teas (Gunning, 1999; Li and Wang, 1998). Preparations of the fresh herb (the above ground portion of the plant) includes *Echinacea* flowers only if the plants are in bloom at the time of harvest. Commercial extracts composed solely of *Echinacea* flowers are not currently available.

A large body of research has been generated regarding the characterization and bioactivity of compounds from *Echinacea* roots and fresh

herb; however, *Echinacea* flowers have not received the same attention, and their bioactivity has not been fully elucidated. The roots of *Echinacea* have been determined to possess cyclooxygenase-1 and –2 inhibitory activity (Clifford et al., 2002) and 5-lipoxygenase inhibitory activity (Müller-Jakic et al., 1994). The majority of research regarding the antioxidant activities of *Echinacea* has focused on root and leaf extracts (Sloley et al., 2000; Hu and Kitts, 2000; Pietta et al., 1998), leaving the antioxidant activity of compounds from *Echinacea* flowers to be determined.

Echinacea spp. possess a diverse assemblage of phenolic antioxidants (Bauer and Wagner, 1991) believed to be responsible for the antioxidant activity of the plant extracts (Facino et al., 1995). Phenolic antioxidants are known to have free radical scavenging properties and antioxidant properties with regard to the oxidation of low-density lipoproteins. The current popularity of natural antioxidants is due to their reported role as modulators of lipid peroxidation as it relates to diseases such as atherogenesis, thrombosis, and carcinogenesis (Frankel, 1999). *Echinacea purpurea* flowers present a new source of such natural antioxidants for the phytoceutical market.

The purpose of this research was to determine the antioxidant and cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activities of compounds isolated from *E. purpurea* flowers and compounds identified previously in *E. purpurea* extracts. Caffeoyl derivatives reported in *E. purpurea* (caffeic acid, caftaric acid, chlorogenic acid, and cichoric acid) (**Figure 1.4**), and flavonoids (quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-

robinobioside) (**Figure 3.3**) and anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside) (**Figure 3.2**) isolated from *E. purpurea* flowers were examined to determine the potential bioactivity of *E. purpurea* flower extracts.

MATERIALS AND METHODS

General Experimental

Anthocyanins cyanidin-3-*O*-β-D glucopyranoside (**8**) and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) and flavonoids quercetin-3-*O*-rutinoside (**10**), kaempferol-3-*O*-robinobioside (**11**), and quercetin-3-*O*-glucoside (**12**) were isolated from methanolic extracts of lyophilized *E. purpurea* (L.) Moench flowers as previously described (**Chapter 3**). Caffeic acid (**13**), caftaric acid (**14**), cichoric acid (**15**), and chlorogenic acid (**16**) were purchased from Sequoia Research Products (Oxford, U.K.).

Cyclooxygenase Inhibitory Assay

An in vitro system was used to assess the cyclooxygenase inhibitory activity of *E. purpurea* flavonoids, anthocyanins, and caffeoyl derivatives. The COX-1 enzyme used in the assay was obtained from ram seminal vesicle microsomal preparations according to previously reported methods (Meade et al, 1993). A recombinant human microsomal preparation of COX-2 was provided by Dr. David Dewitt (Michigan State University, East Lansing, MI). The assay was conducted in an Instech chamber (Instech Laboratory, Plymouth Meeting, PA) maintained at 37 °C, containing 600 μL of a reaction buffer comprised of 0.1 M

Tris, 1 mM phenol, cyclooxygenase enzyme (10 μL), and 17 μg hemoglobin. Flavonoids, anthocyanins, and caffeoyl derivatives were dissolved in DMSO such that a 10 μL aliquot yielded a final concentration of 100, 50, or 10 μg/mL for the caffeoyl derivatives and anthocyanins or 100 μg/mL for the flavonoids. Assay concentrations in μM are noted in **Appendix A**. The reaction was initiated with the addition of arachidonic acid. Initial oxygen uptake was monitored using a YSI model 5300 biological oxygen monitor (Yellow Springs Instruments, Inc., Yellow Springs, OH) and recorded using Quicklog Windows software, version 1.0 (Strawberry Tree, Inc., Sunnyvale, CA). Analyses of samples and controls were performed in triplicate.

Aspirin, ibuprofen, naproxen, celecoxib (Celebrex™), rofecoxib (Vioxx™), and valdecoxib (Bextra™) were dissolved in DMSO and used as controls for both the COX-1 and COX-2 inhibitory assays. Aspirin, ibuprofen, and naproxen controls were assayed at concentration resulting in approximately 50% inhibition of the COX-1 enzyme. Aspirin was tested at 180 μg/mL (1000 μM), ibuprofen at 2.06 μg/mL (10 μM), and naproxen at 2.52 μg/mL (10 μM) for both COX-1 and COX-2 . Celebrex™, Vioxx™, and Bextra™ were obtained as physician's professional samples, ground to a fine powder, and tested as a suspension in DMSO at 1.67 μg/mL for both COX-1 and COX-2. The initial decrease in O_2 levels in the presence of test compounds and standard NSAIDs was compared to that of the DMSO control in order to determine inhibition of COX-1 or COX-2 enzymes.

Antioxidant Assay

The antioxidant assay was conducted according to previously described methods (Arora and Strasburg, 1997). 1-Stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (lipid) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (fluorescent probe) were combined, dried under vacuum, and suspended in MOPS buffer (1 mL of 0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS). Large unilamellar liposomes were prepared by subjecting the lipid-probe mixture to ten freeze-thaw cycles in a dry ice-ethanol bath followed by extrusion through a 100 nm pore size membrane. The final assay volume of 2 mL was composed of 100 μL of HEPES buffer (50 mM HEPES and 50 mM Tris), 200 μL NaCl (1 M), 1.64 mL of N_2-sparged water, 20 μL of sample (to achieve a final concentration of 100, 50, or 10 μg/ml) or DMSO (control), and a 20 μL aliquot of liposome suspension. Peroxidation was initiated by the addition of 20 μL of $FeCl_2 \bullet 4H_2O$ (0.5 mM) and the samples were gently vortexed. Probe fluorescence was monitored using a Turner fluorometer with a narrow bandpass 360 filter and a sharp cut 415 filter at times 0, 1, 3, 6, and every three minutes thereafter for 21 min. Antioxidant activity was determined by comparing the relative fluorescence of test compounds with the control. Test compounds were compared to standard synthetic antioxidants *tert*-butyl hydroquinone (TBHQ, 1.7 μg/mL), butylated hydroxytoluene (BHT, 2.2 μg/mL), and butylated hydroxyanisole (BHA, 1.8 μg/mL), as well as the natural antioxidant vitamin E (4.3 μg/mL). Analyses of samples and controls were performed in triplicate.

RESULTS AND DISCUSSION

Cyclooxygenase Inhibitory Assay

The COX-1 and COX-2 inhibitory activities of compounds from *E. purpurea* are summarized (**Figure 4.1**). The anthocyanins cyanidin-3-*O*-β-D glucopyranoside (**8**) and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) demonstrated the greatest anti-inflammatory activities of the compounds tested, with 28.2 ± 0.3 and 25.9 ± 1.6% inhibition of COX-1 and 25.6 ± 1.1 and 22.9 ± 0.5% inhibition of COX-2, respectively. Weak inhibition of COX-1 by quercetin-3-*O*-rutinoside (**10**) and quercetin-3-*O*-glucoside (**12**) was noted, with inhibitory activities of 14.5 ± 7.8 and 11 ± 5.6%, respectively (**Figure 4.1**). Kaempferol-3-*O*-robinobioside (**11**) did not inhibit COX-1. None of the flavonoids tested exhibited COX-2 inhibitory activity. Caffeic acid (**13**), caftaric acid (**14**), chicoric acid (**15**), and chlorgenic acid (**16**) demonstrated nearly negligible COX-1 inhibitions of 5.1 ± 1.0, 5.9 ± 1.0, 8.9 ± 1.0, and 7.8 ± 0.9%, respectively (**Figure 4.1**). COX-2 inhibitory activity for the caffeoyl derivatives was weak, and compounds **13-16** were not considered active inhibitors of this enzyme.

Cyanidin-3-*O*-β-D glucopyranoside (**8**) and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) demonstrated similar dose-response effects against COX-1 and COX-2 at 100, 50, and 10 μg/mL (**Figure 4.2**). At 50 and 10 μg/mL, both compounds were effective inhibitors of COX-1; however, COX-2 inhibition was significant only at 50 μg/mL. While the flavonoids and caffeoyl derivatives were

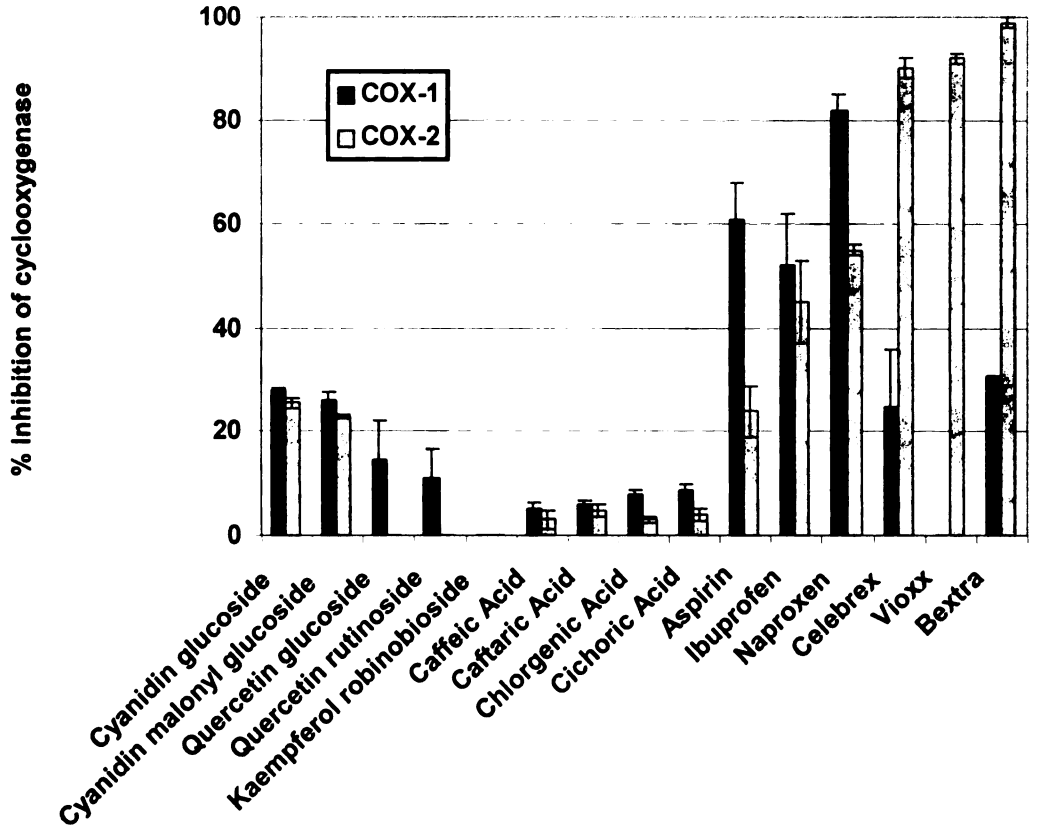


Figure 4.1. Comparison of cyclooxygenase-1 and –2 inhibitory activities for compounds from *E. purpurea*. Compounds were assayed at 100 μg/mL and compared to NSAID standards aspirin (180 μg/mL), ibuprofen (2.06 μg/mL), naproxen (2.52 μg/mL), Celebrex™ (1.67 μg/mL), Vioxx™ (1.67 μg/mL) and Bextra™ (1.67 μg/mL). Data represent the average ± one standard deviation.

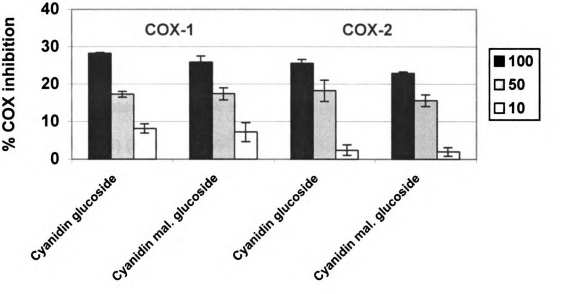


Figure 4.2. COX-1 and COX-2 inhibitory activities for anthocyanins isolated from *E. purpurea* flowers at 100, 50 and 10 μg/mL. Numbers in the legend represent the concentration assayed in μg/mL. Data represent the average ± one standard deviation.

also tested at 50 and 10 μg/mL, only the anthocyanins were active at concentrations below 100 μg/mL against either COX-1 or COX-2.

Antioxidant Assay

The results for the antioxidant assay are expressed as the mean percent inhibition of oxidation following 21 minutes of incubation (**Figure 4.3**). When tested at 10 μg/mL, cyanidin-3-*O*-β-D glucopyranoside (**8**) (81% inhibition) demonstrated greater antioxidant activity than cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) (33% inhibition) after 21 min. Caffeic acid (**13**), caftaric acid (**14**), cichoric acid (**15**), chlorogenic acid (**16**) exhibited strong antioxidant activities of 73, 35, 79, and 73%, respectively.

At 100 μg/mL, quercetin-3-*O*-rutinoside (**10**), kaempferol-3-*O*-robinobioside (**11**), and quercetin-3-*O*-glucoside (**12**) exhibited good antioxidant activity (73, 83, and 85%, respectively) when compared to the synthetic antioxidants and vitamin E (**Figure 4.4**). Reducing the concentration to 50 μg/mL still resulted in significant antioxidant activity for compounds **10**, **11**, and **12**, with inhibitions of 47, 54, and 42%, respectively (**Figure 4.3**). Compounds **10** and **12** exhibited antioxidant activity at 10 μg/mL superior to vitamin E at the end of 21 minutes, with inhibitions of 54 and 42%, respectively. Activity for compound **11** decreased to 3% at 10 μg/mL.

Additional tests were performed by combining 5 μg/mL aliquots of the flavonoids from *E. purpurea* in various combinations to yield a 10 μg/mL mixture. Inhibitions of 57, 52, and 16% were noted for the mixtures of compounds **10/12**,

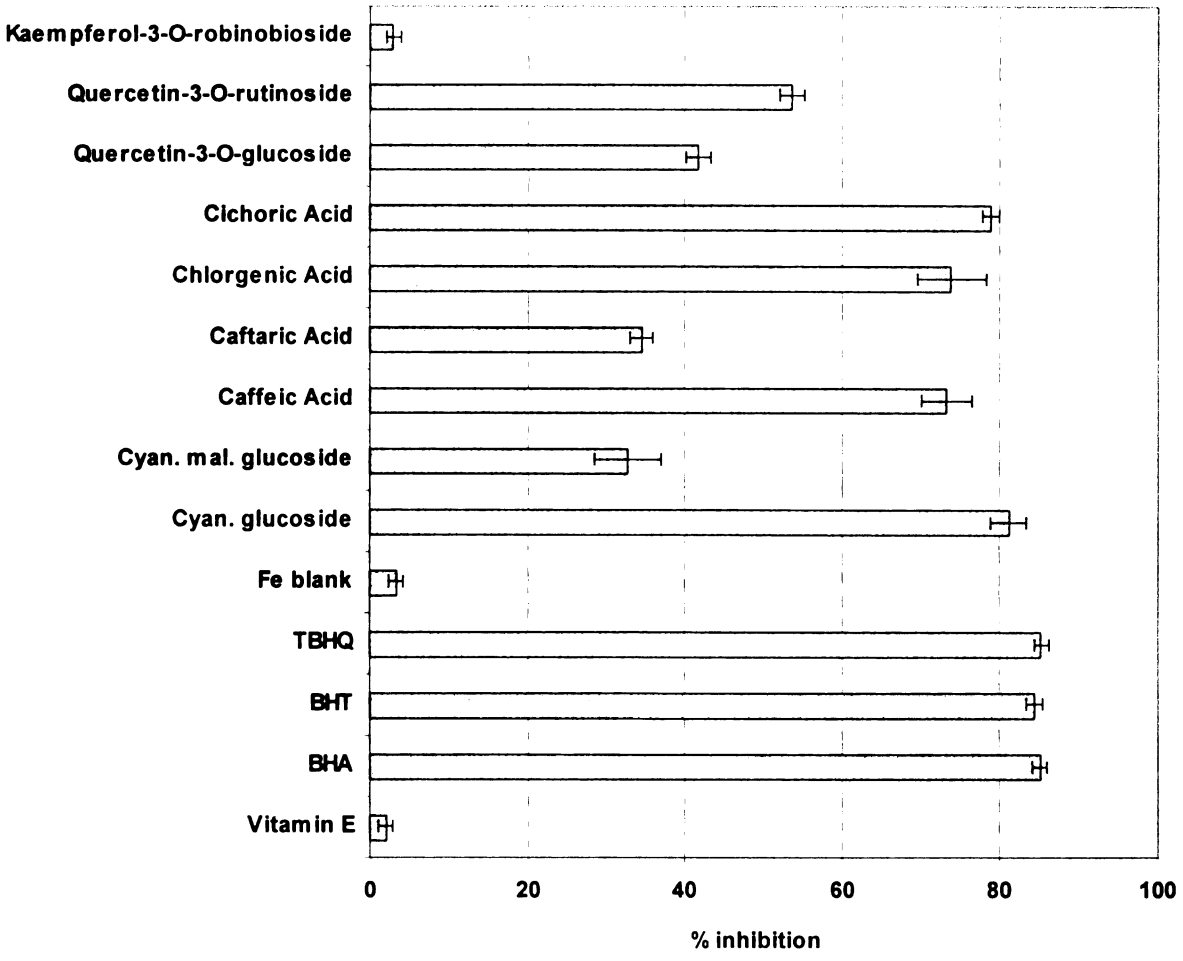


Figure 4.3. Antioxidant activity exhibited by compounds from *E. purpurea* assayed at 10 μg/mL and compared to oxidation exhibited by Fe^{2+} control. Activity was calculated relative to the DMSO control and compared to standard synthetic antioxidants TBHQ (1.7 μg/mL), BHT (2.2 μg/mL), and BHA (1.8 μg/mL), as well as the natural antioxidant vitamin E (4.3 μg/mL). Data represent the average ± one standard deviation.

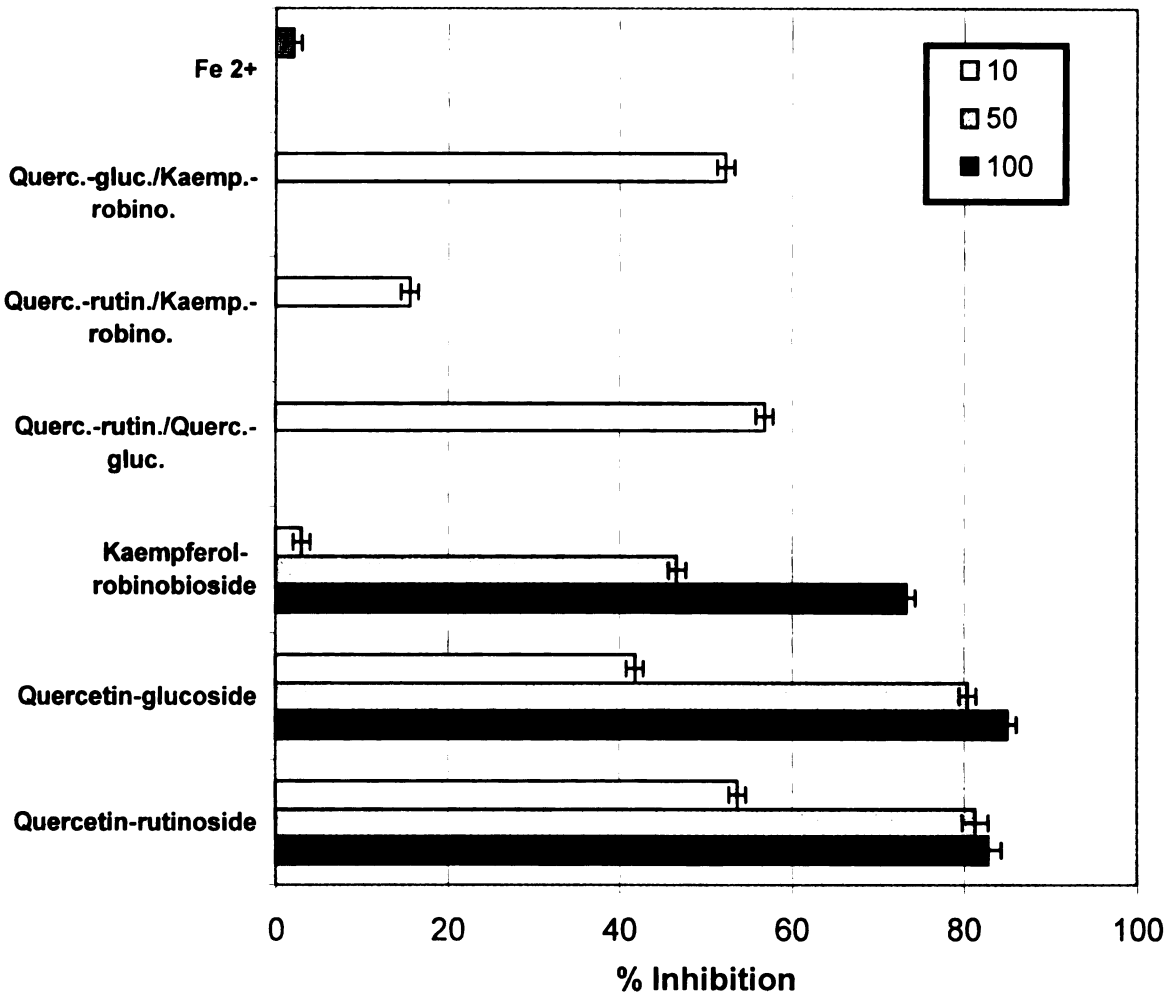


Figure 4.4. Antioxidant activity at 21 minutes for quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside at 10, 50, and 100 μg/mL compared to oxidation exhibited by Fe^{2+} control. Activity was calculated relative to the DMSO control. Numbers in the legend represent the concentration assayed in μg/mL. Data represent the average ± one standard deviation.

11/12, and **10/11**, respectively. Activity was lower for the mixture of compounds **10** and **11**, with an inhibition of 16% as compared to 54% for quercetin-3-*O*-rutinoside and 3% for kaempferol-3-*O*-robinobioside when tested individually. When examined at a concentration of 10 μg/mL, the combination of compounds **11** and **12** demonstrated greater activity (52% inhibition) than compound **12** (42%) or compound **11** (3%). These results suggest a possible synergistic effect between kaempferol-3-*O*-robinobioside (**11**) and quercetin-3-*O*-glucoside (**12**) in the antioxidant assay.

Caffeoyl derivatives (**Figure 1.4**) are considered to be among the most active components of *Echinacea* spp. (Li and Wang, 1998). For example, cichoric acid, one of the major compounds in the fresh herb of *E. purpurea*, has numerous documented biological effects (Nüsslein et al., 2000). Unfortunately, caffeoyl derivatives are highly susceptible to enzymatic and oxidative degradation during extraction (Bergeron et al., 1999). Cichoric acid is rapidly degraded by polyphenol oxidase in fresh plant juice and is a challenge to isolate due to its instability. Lyophilization has been shown to drastically reduce cichoric acid and caftaric acid quantities in *Echinacea* (Kim et al., 2000b). In order to avoid degradation during the processing of fresh *E. purpurea* flowers, caffoyl derivatives were purchased rather than isolated for examination in the cyclooxygenase inhibitory and antioxidant assays.

Previous work regarding the anti-inflammatory activity of *Echinacea* has focused on root extracts rather than whole plant or flower extracts. Alkamides isolated from *E. angustifolia* roots have demonstrated cyclooxygenase and 5-

lipoxygenase inhibitory activities in in vitro assays (Müller-Jakic et al., 1994). An examination of the alkamides isolated from *E. purpurea* roots revealed their comparative COX-1 and COX-2 inhibitory activities (Clifford et al., 2002). The anti-inflammatory activities of *Echinacea* flower extracts or compounds isolated from *E. purpurea* flowers have not been previously determined.

Anthocyanins are known inhibitors of COX-1 and COX-2 enzymes (Wang et al., 1999; Seeram et al., 2001). The results obtained here regarding the COX-1 and COX-2 inhibitory activities of cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside, isolated from *E. purpurea* flowers, are in agreement with those previously reported. Cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside possessed slightly lower activity than cyanidin-3-*O*-β-D glucopyranoside at a concentration of 100 μg/mL, confirms previous research suggesting that the activity is due to the cyanidin aglycone (Wang et al., 1999).

Neither the flavonoids nor the caffeoyl derivatives demonstrated significant anti-inflammatory activity in the COX-1 or COX-2 inhibitory assays. Many whole plant and root extracts are standardized to echinacoside content or total phenolic content. *Echinacea purpurea* extracts do not contain echinacoside, and the caffeoyl derivatives and flavonoids examined in the cyclooxygenase inhibitory assays exhibited very weak activity. In order to obtain significant anti-inflammatory effects from *E. purpurea* flower or fresh herb extracts, the anthocyanins examined in this study may provide a better standardization marker to ensure activity.

The antioxidant activity of *Echinacea* extracts and compounds isolated from *Echinacea* have been evaluated in various in vitro assays. A phenolic *Echinacea* extract (plant species, part, and extraction method not identified), standardized to 4.5% echinacoside, was found to be ineffective at scavenging free radicals by the Trolox equivalent antioxidant assay (Pietta et al., 1998). Caffeic acid, chlorogenic acid, and cichoric acid have been assessed for their ability to prevent free radical-induced degradation of collagen, demonstrating that cichoric acid exhibited the most pronounced protective effects (Facino et al., 1995). These results suggest that the testing of specific compounds from *Echinacea* extracts rather than complex mixtures might yield important information regarding antioxidant activity. The use of echinacoside as a phenolic standardization marker should also be reconsidered due to the lack of significant occurrence in *E. purpurea* extracts.

Antioxidant studies of *Echinacea* have focused on the roots and leaves of the plant, overlooking extracts of the flowers. *Echinacea purpurea* root and leaf extracts were found to be effective in scavenging hydroxyl free radicals in an assay measuring the peroxidase-catalyzed accumulation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sloley et al., 2000). Methanolic *E. purpurea*, *E. angustifolia*, and *E. pallida* root extracts also suppressed hydroxyl radical generation by 20-34% due to the copper chelating capacity of these extracts (Hu and Kitts, 2000). The ability of *E. purpurea* root and leaf extracts to inhibit Fe^{2+}-induced lipid peroxidation in a catecholaminergic neuroblastoma SH-SY5Y cells using a thiobarbituric acid assay was assessed, with leaf extracts demonstrating

higher antioxidant activity than root extracts (Sloley et al., 2000). Based upon the antioxidant potential demonstrated by previous research and the antioxidant results obtained in these studies, compounds isolated from *E. purpurea* flowers possess significant antioxidant activity.

Previous research regarding the antioxidant potential of various flavonoids suggested that the ability to scavenge free radicals and chelate metal ions stemmed from the degree of conjugation, the presence and arrangement of substituents on the B-ring, and substitution at the 3 position on the C-ring of the flavonoid molecule. Compared to quercetin, kaempferol lacks the 3'-hydroxyl group on the B-ring. Research has suggested that the 3'-hydroxyl group of quercetin contributes to the chelation and antioxidant potential of the molecule (Rice-Evans, 1999). When tested at 10 μg/mL, the lower antioxidant activity observed for compound **11** compared to compounds **10** and **12** supported these previously reported observations of the importance of the 3'-hydroxyl group as it pertains to antioxidant activity.

The results from the cyclooxygenase inhibitory and antioxidant assays indicate that *E. purpurea* flowers are a potential source of bioactive compounds. Currently, *E. purpurea* flowers are included in extracts of the fresh herb only if the flower is in bloom at the time of harvest. The results presented here demonstrate that extracts prepared solely from the flowers may provide a concentrated source of bioactive compounds, based upon the cyclooxygenase inhibitory and antioxidant activities of the anthocyanins, flavonoids, and caffeoyl derivatives examined in these studies. The promising results obtained in this work indicate

that further research regarding the development of an *E. purpurea* flower-based natural product supplement is warranted.

CHAPTER 5.

CHARACTERIZATION OF CYTOKINE PRODUCTION IN RAW 264.7 MURINE MACROPHAGES FOLLOWING TREATMENT WITH COMPOUNDS FROM *ECHINACEA PURPUREA* (L.) MOENCH

ABSTRACT

Echinacea phytoceuticals are consumed for purported prevention of or reduction in the severity of upper respiratory tract infections; however, little is known regarding their immune mode of action. Flavonoids (quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) and anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside), isolated from methanolic extracts of lyophilized *E. purpurea* flowers, and alkamides isolated *Echinacea purpurea* (L.) Moench roots (undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide, undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide) were examined for their effects upon the production of cytokines IL-6 and TNF-α in murine macrophages. Caffeic acid, caftaric acid, chlorogenic acid, and cichoric acid, reported components of *E. purpurea*, were also tested. Cytotoxicity was assessed concurrently by using the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and the highest concentration examined for all four alkamides, 100 μg/mL, adversely affected cells and was not used in the macrophage assay. When cells were

treated solely with compounds from *E. purpurea*, no induction of IL-6 or TNF-α occurred. Co-treatment of cells with 100 or 1000 ng/mL lipopolysaccharide (LPS) demonstrated that several compounds isolated from *E. purpurea* assayed at concentrations of 1, 10, 50, and 100 μg/mL have the ability to suppress induced inflammatory response. The alkamides demonstrated the greatest ability to affect IL-6 and TNF-α production in cells stimulated with 100 and 1000 ng/mL LPS, with significant reductions of IL-6 and TNF-α noted when treated with as little as 1 μg/mL. The anti-inflammatory activity of the alkamides reported here supports the anecdotal claims of pain and fever relief associated with *Echinacea* use. No significant reductions in IL-6 were noted for either the anthocyanins or flavonoids when stimulated with 100 or 1000 ng/mL LPS; however, reductions in TNF-α levels were noted with 10, 50, and 100 μg/mL quercetin-3-*O*-rutinoside and at 1, 10, 50, and 100 μg/mL cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside. The trend in IL-6 and TNF-α production indicated a potential synergistic effect of the caffeoyl derivatives with LPS. Co-treatment of the macrophages with the caffeoyl derivatives and 100 and 1000 ng/mL LPS resulted in significant increases in IL-6 and TNF-α levels at 50 and 100 μg/mL. These results demonstrate that individual compounds from *E. purpurea* might possess opposing activities with regard to immune stimulation and/or suppression, and might explain the disparity previously reported in clinical studies.

INTRODUCTION

Echinacea phytoceuticals are among the most popular natural product supplements in the United States (Barrett et al., 1999). Historically, *Echinacea* root and fresh herb extracts were used for the treatment of fevers, burns, insect bites, wounds, coughs, and to alleviate the pain of toothaches, headaches, and stomach cramps (Bauer and Wagner, 1991). The current popularity of *Echinacea* is due primarily to reported anecdotal claims of antiviral and nonspecific immunostimulatory properties (Borchers et al., 2000; Proksch and Wagner, 1987).

A limited number of studies have been conducted to substantiate the anecdotal claims associated with *Echinacea* use (Borchers et al., 2000). The pain relieving and general immune stimulating effects of *Echinacea* have been evaluated to some extent (Bauer, 1998; Bauer and Wagner, 1991; Proksch and Wagner, 1987). 4-*O*-Methyl-glucuronoarabinoxylan, isolated from *E. purpurea*, was believed to be responsible for enhancing phagocytosis in an in vitro granulocyte assay (Proksch and Wagner, 1987). In another study, mouse L-929 cells treated with crude aqueous and methanol extracts of *Echinacea* whole plant exhibited 50-80% resistance to herpes, influenza, and vesicular stomatitis viruses (Wacker and Hilbig, 1978). In addition, caffeic acid and cichoric acid, isolated from *Echinacea*, have demonstrated the ability to reduce the infectivity of vesicular stomatitis virus by more than 50% at 62.5 μg/ml of caffeic acid or 125 μg/ml of cichoric acid in mouse L-929 cells. When incubated with the caffeoyl

derivatives, infectivity was reduced by more than 50% (Cheminat et al., 1988). Acidic arabinogalactan, produced by *E. purpurea* cell cultures, activated macrophages against *Leishmania enriettii* and tumor cells, decreasing *L. enriettii* growth in intracellular and phagocytosis-associated assays (Luettig et al., 1989).

A variety of studies have been performed to determine the anti-inflammatory activity of extracts and compounds from *Echinacea*. Intravenous and topical administration of aqueous *E. angustifolia* root extracts have been shown to inhibit carrageenan-induced paw inflammation in mice (Tragini et al., 1988; Tragini et al., 1985). Alkamides from *E. angustifolia*, when examined in vitro, were found to possess cyclooxygenase and 5-lipoxygenase inhibitory activities (Müller-Jakic et al., 1994). The cyclooxygenase-1 and cyclooxygenase-2 inhibitory activities of alkamides from *E. purpurea* have been compared (Clifford et al., 2002). *Echinacea purpurea* alkamides demonstrated in vitro inhibition of both enzyme isoforms; however, preferential COX-2 inhibition was not identified.

Several *Echinacea* products, including *E. purpurea* roots and herb, *E. purpurea* and/or *E. angustifolia* whole plant extracts standardized either to 4% phenolic content (chlorogenic acid and cichoric acid) or to echinacosides/alkamides, and *Echinacea* pressed juice preparations, were examined to determine IL-6 and TNF-α production in LPS-stimulated murine macrophages (Rininger et al., 2000). Quantitative determination of IL-6 and TNF-α indicated that only the *E. purpurea* root and herb materials, subjected to a simulated digestion (incubation with various simulated gastric fluids), exhibited

macrophage activating activity based upon increased cytokine production. Standardized extracts and samples not subjected to the digestion protocol did not demonstrate significant immunomodulatory activity (Rininger et al., 2000).

Clinical studies have analyzed the effects of various *Echinacea* preparations for the prevention and/or treatment of colds and upper respiratory tract infections (Bauer, 1998). *Echinacea pallida* and *E. purpurea* root extracts significantly reduced the duration of respiratory tract infections (Bräunig et al., 1993; Bräunig et al., 1992). Administration of a dried, ethanol extract of *E. purpurea* (95% herb and 5% root) in tablet form reduced the symptoms of respiratory tract infections (Brinkeborn et al., 1998). *Echinacea purpurea* fresh herb juice has demonstrated a prophylactic effect in preventing the development of upper respiratory tract infections (Hoheisel et al., 1997).

While the studies indicated above demonstrate beneficial effects of *Echinacea* extracts, many other clinical trials found no significant effects on the duration or severity of colds and respiratory tract infections associated with the consumption of *Echinacea* extracts (Bauer, 1998; Gunning, 1999). *Echinacea purpurea* juice did not significantly reduce the number or duration of upper respiratory tract infections in patients with a prior history of upper respiratory tract infections (Schöneberger, 1992). In another clinical trial, *E. purpurea* and *E. angustifolia* root extracts did not significantly prevent upper respiratory tract infections (Melchart et al., 1998). No significant differences were noted with regard to the incidence, duration, or severity of colds and respiratory tract

infections in a study designed to determine the prophylactic effect of expressed *E. purpurea* juice (Grimm and Müller, 1999).

The disparity reported regarding the prevention and treatment of upper respiratory tract infections by *Echinacea* extracts may result from dissimilar chemical compositions of crude extracts examined. The variability in the composition of the extracts was the result of a lack of uniform, industry-wide standardization methods. The difference in extraction method and reported differences between *Echinacea* species contributed to the variability in *Echinacea* extracts examined (Binns et al., 2002). Testing of specific compounds from *Echinacea* would provide a chemical basis upon which the standardization of *Echinacea*-based herbal supplements could be based.

The purpose of this research was to determine the effects of co-treating murine macrophage cells (RAW 264.7) with lipopolysaccharide (LPS), an inflammagenic stimulus, and compounds isolated from *E. purpurea*. The compounds examined in this study included *E. purpurea* anthocyanins **8** and **9** (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside), flavonoids **11** and **12** (quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside), caffeoyl derivatives **13-16** (caffeic acid, caftaric acid, cichoric acid, and chlorogenic acid), and alkamides **1**, **2**, **4**, and **6/7** (undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide, undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide). Anti-inflammatory effectiveness

was noted by the reduction in interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) produced by the cells stimulated with LPS compared to control values. MTT assays were performed for each compound concomitant with the murine macrophage assay to assess potential cytotoxicity of the doses examined.

MATERIALS AND METHODS

General Experimental

Alkamides undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide (**1**), undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide (**2**), undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide (**4**), and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**6/7**) were isolated from *E. purpurea* roots as previously described (**Chapter 2**). Anthocyanins cyanidin-3-*O*-β-D glucopyranoside (**8**) and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside (**9**) and flavonoids quercetin-3-*O*-rutinoside (**10**) and quercetin-3-*O*-glucoside (**12**) were isolated from methanolic extracts of lyophilized *E. purpurea* (L.) Moench flowers as previously described (**Chapter 3**). Caffeic acid (**13**), caftaric acid (**14**), cichoric acid (**15**), and chlorogenic acid (**16**) were purchased from Sequoia Research Products (Oxford, U.K.).

Cell Culturing

Murine macrophage cells (RAW 264.7, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS)

Atlanta Biologicals, Norcross, GA), 1 mM sodium pyruvate (Sigma), 1% (v/v) NCTC-135 (Gibco Laboratories, Chagrin Falls, IL) streptomycin (100 μg/mL, Sigma), and penicillin (100 U/mL, Sigma). Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. For the assay, cells were grown to confluency in sterile tissue culturing dishes, the media was removed, and the cells were incubated for 10 min with phosphate buffered saline (PBS). Cells were detached by gentle, repeated pipetting, centrifuged, and resuspended in fresh media. Cell viability and number were assessed by trypan blue dye exclusion (Strober, 1991) and enumerated using a hemacytometer. Three cell stock solutions were prepared at a cell density of 5 x 10^5 cells/mL with 0, 200, or 2000 ng/mL lipopolysaccharide (LPS) (Sigma, SI 3.6 @ 15.6 μg/mL LPS, 1.5 EU/ng LPS). Compounds **1**, **2**, **4**, **6/7**, **8-9**, **10**, **12**, and **13-16** were dissolved in DMSO and added to media to yield stock test compound concentrations of 2, 20, 100, and 200 μg/mL. Final DMSO concentrations did not exceed 0.02% DMSO in the test compound stocks.

Cell stocks with LPS (0, 200, and 2000 ng/mL LPS) (400 μL) were added to sample wells of a 48-well plate (Costar, Cambridge, MA) in combination with 400 μL of the test compound stock solutions (2, 20, 100, and 200 μg/mL). Final assay concentrations were 0,100, and 1000 ng/mL LPS and 0, 1, 10, 50, and 100 μg/mL test compound, with a final cell density of 1 x 10^6 cells/mL. Assay concentrations in μM are noted in **Appendix A**. Vehicle controls with equivalent concentrations of DMSO were also prepared. Cultures containing controls and test compounds were incubated for 12 h, after which time the media was

immediately aspirated from each well and stored at -80 °C in cryogenic vials. All compounds and controls were prepared in triplicate.

MTT Assay

The MTT assay determines adverse effects on cells through the cleavage of the tetrazolium ring in the water-soluble tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenase enzymes in live cells to form an insoluble formazan (Marshall et al., 1995; Denizot et al., 1986; Mossman, 1983). Dead cells cannot convert MTT, therefore spectroscopic measurement of the dissolved formazan yields valuable information regarding the potential cytotoxic effects of test compounds. Reductions in IL-6 and TNF-α levels might be incorrectly attributed to the action of the compounds on the inflammation response rather than the underlying cell death that resulted in fewer cells capable of producing the cytokines. The MTT assay ensures that the effects noted in the cell assay were not due to overt toxicity of the compounds tested.

The MTT assay was performed concurrently with the cell culture assay. The MTT cleavage assay was performed as previously described (Ji et al., 1998) with minor modifications. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) reagent was prepared by dissolving the dye in 0.01 M PBS and filter sterilized to yield a 5 mg/mL stock solution. Aliquots of 100 μL each of cell stocks and test compounds or controls were added to the wells of a 96-well plate (Costar).

Following an incubation period of 10 h at 37 °C, 50 μL of the MTT stock solution was added to each well, and the plate was further incubated for 2 h at 37 °C. The plates were then centrifuged at 500 x g for 20 min, and the supernatant removed by aspiration. The formazan crystals at the bottom of the plate wells were dissolved by the addition of 200 μL/well DMSO. After the crystals had completely dissolved, the absorbance of each well was read on a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) at λ 570-690 nm. Data management was performed using Vmax Softmax Pro Software version 3.0 (Molecular Devices).

ELISA Quantification of IL-6 and TNF-α

IL-6 and TNF-α produced in the macrophage assay were quantified by enzyme linked immuno-sorbent assay (ELISA) according to previously described procedures (Ji et al., 1998). Microtiter strip wells (Immunolon IV Removawell, Dynatech Laboratories Inc., Chantilly, VG) were coated with 50 μL/well of 1 μL/mL purified rat anti-mouse IL-6 or TNF-α antibodies (BD-PharMingen, San Diego, CA) in 0.1 M $NaHCO_3$ (pH 8.2) and held at 4 °C for 16 h. Wells were washed three times with PBST (0.01 M PBS with 0.2% (v/v) Tween 20) and blocked to prevent nonspecific protein binding with 300 μL/well of 3% bovine serum albumin (BSA) in PBST (0.01 M PSB with 0.2% (v/v) Tween 20) (BSA-PBST) at 37 °C for 30 min. Wells were washed four times, and recombinant murine IL-6 or TNF-α standards (BD-PharMingen) prepared in 10% (v/v) FBS-DMEM and samples from the macrophage assay (recovered media) were added in 50 μL aliquots and incubated for 1 h at 37 °C. After the incubation, the wells

were washed with PBST four times followed by one wash with dH_2O. Aliquots of 50 μL of biotinylated rat anti-mouse IL-6 or TNF-α (BD-PharMingen), diluted to 1 μL/mL in 3% BSA-PBST (w/v), were added to each well and plates were incubated at room temperature for 1 h. Wells were washed six times with PBST and once with dH_2O following the incubation time. After washing, 50 μL of 1.5 μg/mL streptavidin-horseradish peroxidase (Sigma) in 3% BSA-PBST were added to each well. After 1 h incubation at room temperature, wells were washed eight times with PBST and twice with dH_2O. Aliquots of 100 μL of tetramethylbenzidine (TMB) substrate (K-blue Max®, Neogen, Lexington, KY) were added to each well. After the development of color, approximately 15 min, the reaction was stopped with the addition of 100 μL of 6 N H_2SO_4 to each well. The optical density of the sample wells was immediately measured on a Vmax Kinetic Microplate Reader, using Vmax Softmax Pro Software version 3.0 (Molecular Devices) at λ 450 nm.

Statistical Analysis

Data for the cell culture assay were analyzed by one way analysis of variance (ANOVA) followed by Bonferroni's test using SigmaStat for Windows, version 2.0 (Jandel Scientific, San Rafael, CA). The cell culture assay was performed in triplicate, and the media from each cell culture well was analyzed in duplicate. Data from IL-6 and TNF-α quantification are reported as the mean ± one standard deviation. Values in subsequent graphs significantly different from controls ($p<0.05$) were noted by an asterisk (*).

RESULTS AND DISCUSSION

MTT Assay

The MTT assay was performed to determine the potential cytotoxicity of compounds from *E. purpurea* tested in the macrophage assay. Cells were treated with MTT after 10 h of incubation, and incubated for another 2 h to yield a total exposure time equal to that of the macrophage cell assay. No significant differences ($p < 0.05$) were noted between controls (0, 100, and 1000 ng/mL LPS) versus the concentrations tested for compounds **8-9, 10, 12, and 13-16**. The results from the MTT assay indicated that the highest concentration tested for the four alkamides (**1**, **2**, **4**, and **6/7**), 100 μg/mL, adversely affected the macrophage cells (**Figures 5.1-5.4**). Therefore, the 100 μg/mL concentration was not used to determine the effects on cytokine production for compounds **1**, **2**, **4**, and the mixture of **6/7**.

Cytokine Production

Macrophages were treated with compounds from *E. purpurea* or co-treated with LPS and *E. purpurea* compounds to determine effects on cytokine levels. Cytokine induction and/or suppression was determined by ELISA quantification of IL-6 and TNF-α. Results of the cell assays are summarized in **Figures 5.5 – 5.16**. None of the compounds examined demonstrated the ability to directly stimulate RAW 264.7 murine macrophage cells to produce IL-6 or TNF-α in the absence of LPS. Lack of induction without LPS stimulation indicates that the compounds examined did not possess strong immune-

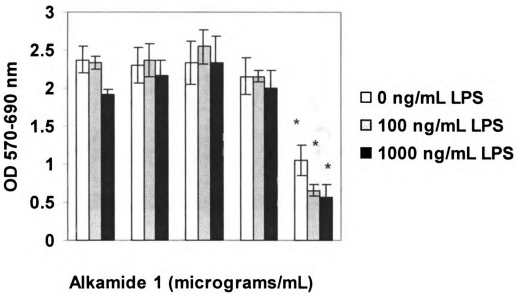


Figure 5.1. MTT assay results for RAW 264.7 macrophages treated with 1, 10, 50, or 100 μg/mL of alkamide **1** from *E. purpurea*. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). Results are representative of two independent studies.

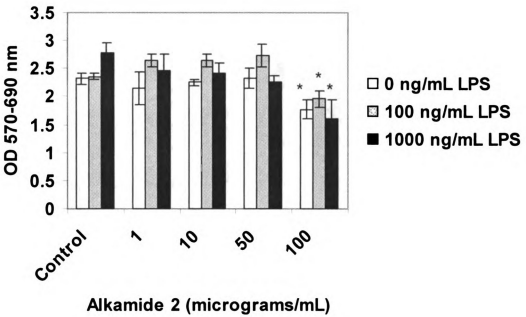


Figure 5.2. MTT assay results for RAW 264.7 macrophages treated with 1, 10, 50, or 100 μg/mL of alkamide **2** from *E. purpurea*. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). Results are representative of two independent studies.

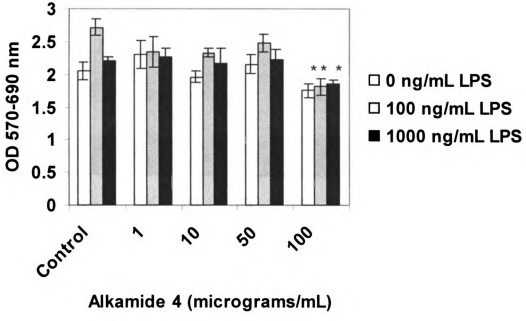


Figure 5.3. MTT assay results for RAW 264.7 macrophages treated with 1, 10, 50, or 100 μg/mL of alkamide **4** from *E. purpurea*. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). Results are representative of two independent studies.

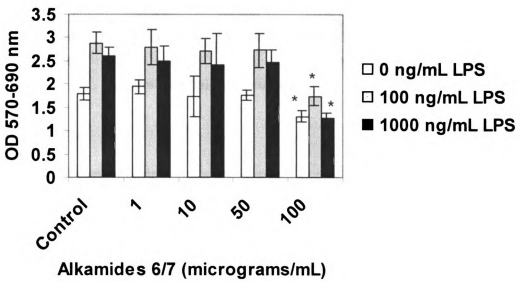


Figure 5.4. MTT assay results for RAW 264.7 macrophages treated with 1, 10, 50, or 100 μg/mL of alkamides **6/7** from *E. purpurea*. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). Results are representative of two independent studies.

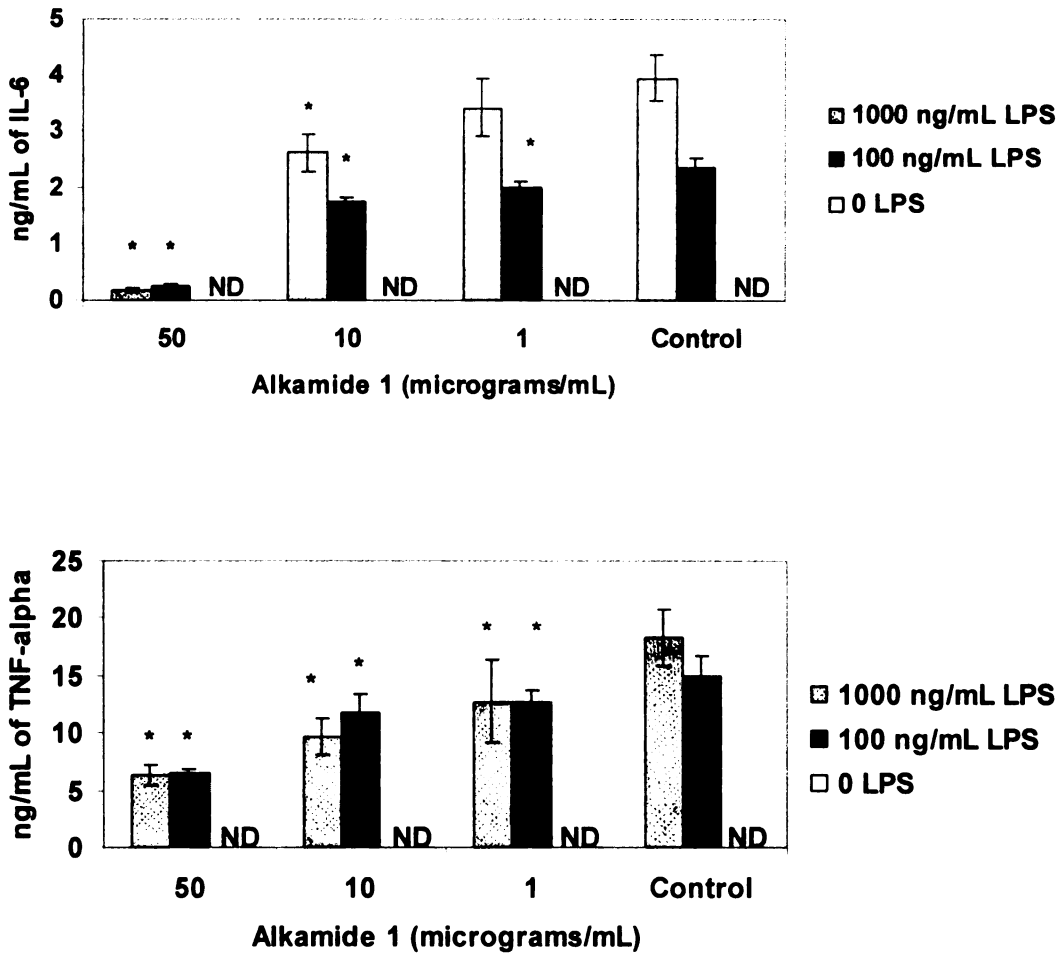


Figure 5.5. Effects of co-treatments of 50, 10, or 1 μg/mL of alkamide **1** with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

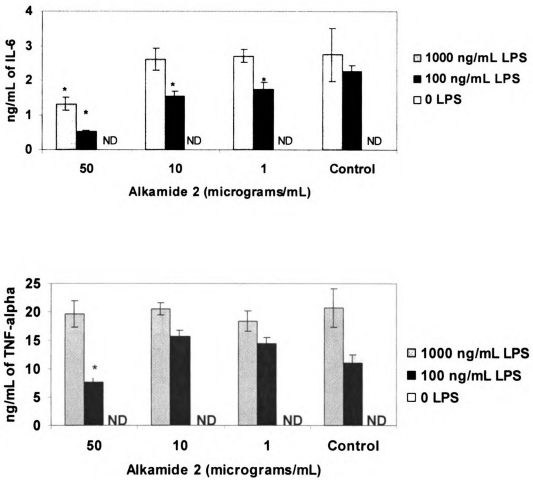


Figure 5.6. Effects of co-treatments of 50, 10, or 1 μg/mL of alkamide **2** with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

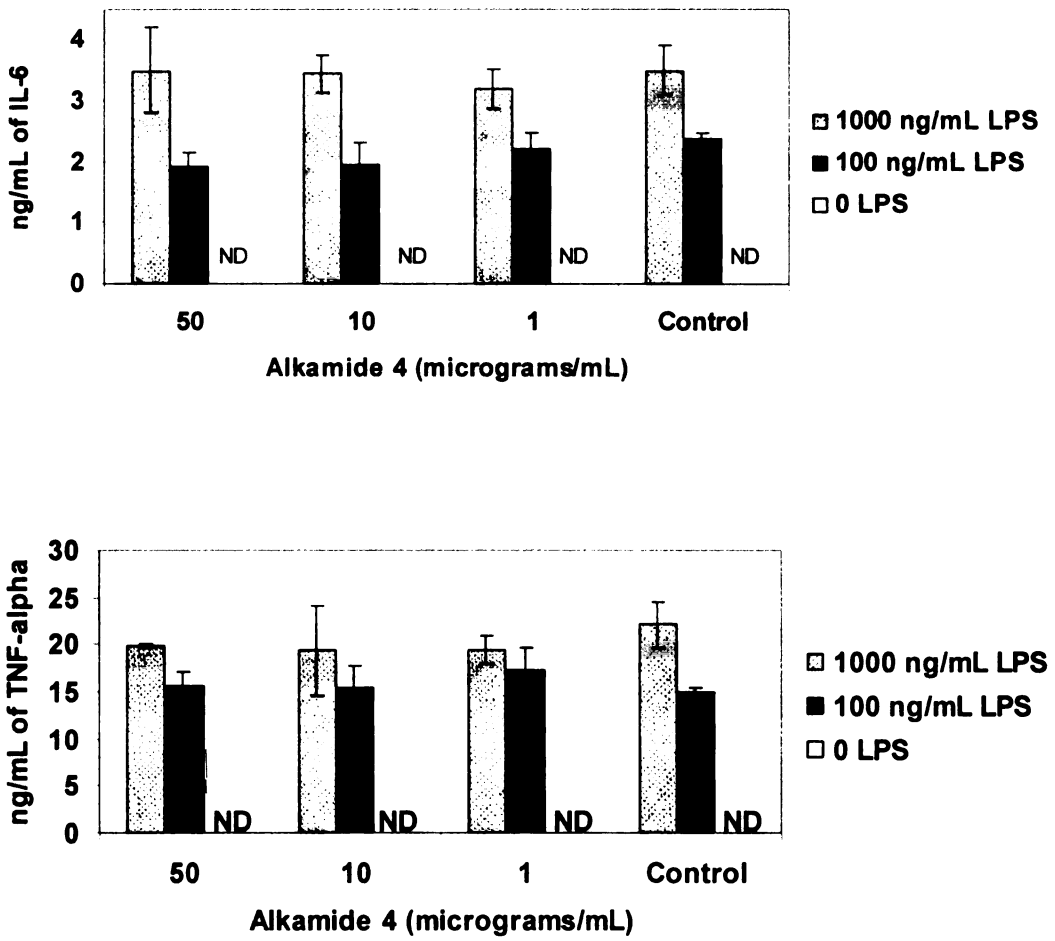


Figure 5.7. Effects of co-treatments of 50, 10, or 1 μg/mL of alkamide **4** with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

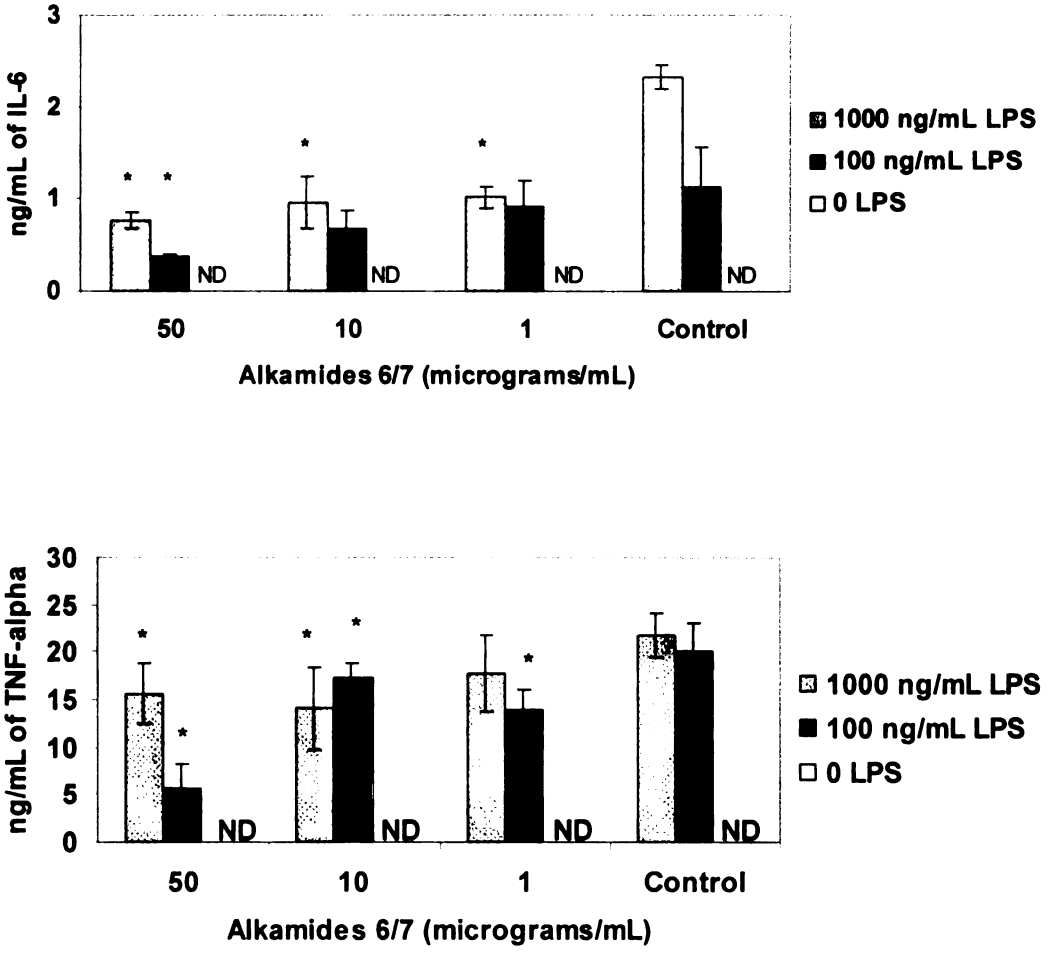


Figure 5.8. Effects of co-treatments of 50, 10, or 1 μg/mL of alkamides **6/7** with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

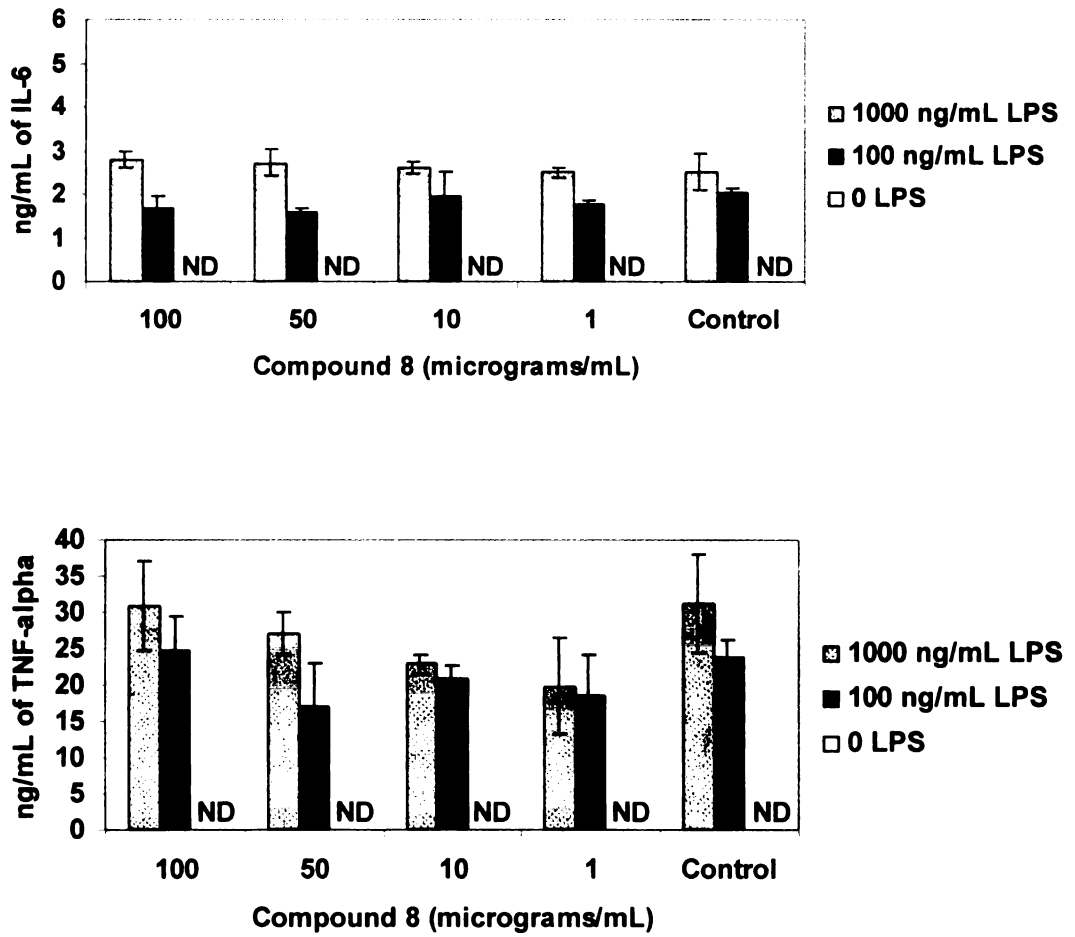


Figure 5.9. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of cyanidin-3-O-glucoside (**8**) with LPS on IL-6 and TNF-α by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

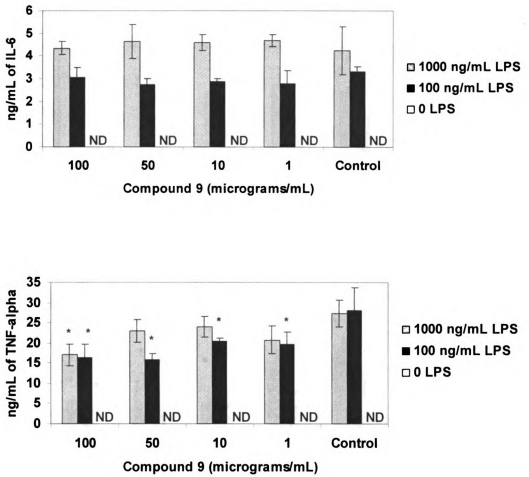


Figure 5.10. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of cyanidin-3-O-malonyl-(1→6)-β-D glucopyranoside (**9**) with LPS on IL-6 and TNF-α by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

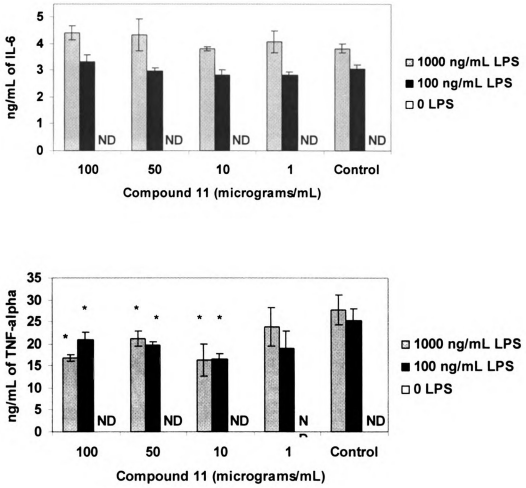


Figure 5.11. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of quercetin-3-O-rutinoside (**11**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

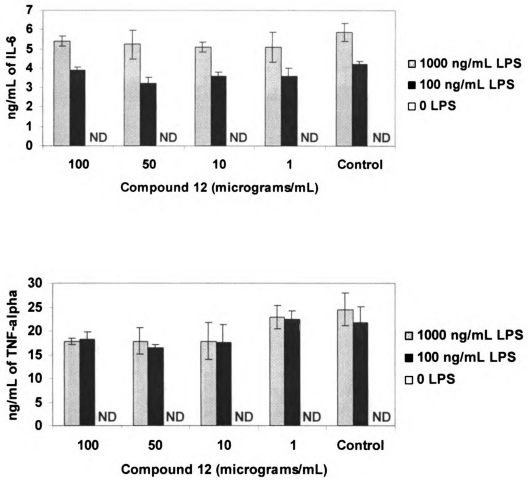


Figure 5.12. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of quercetin-3-O-glucoside (**12**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

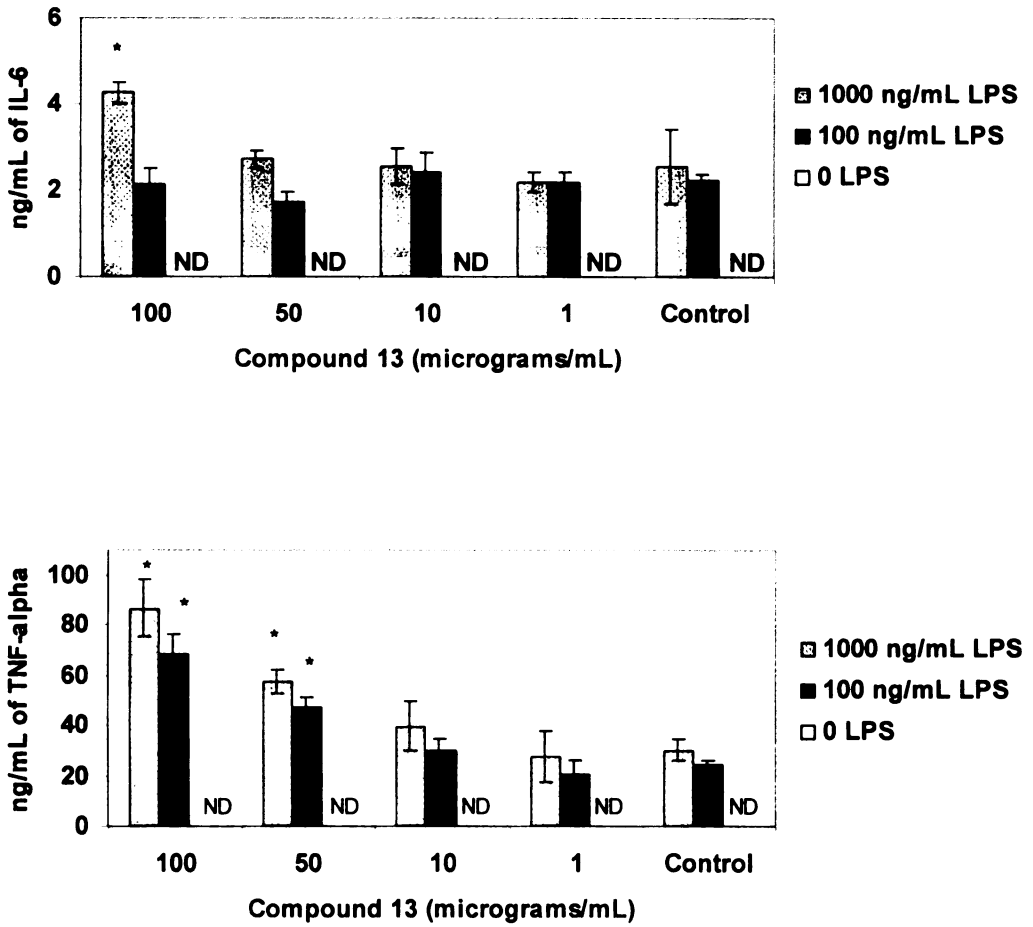


Figure 5.13. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of caffeic acid (**13**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

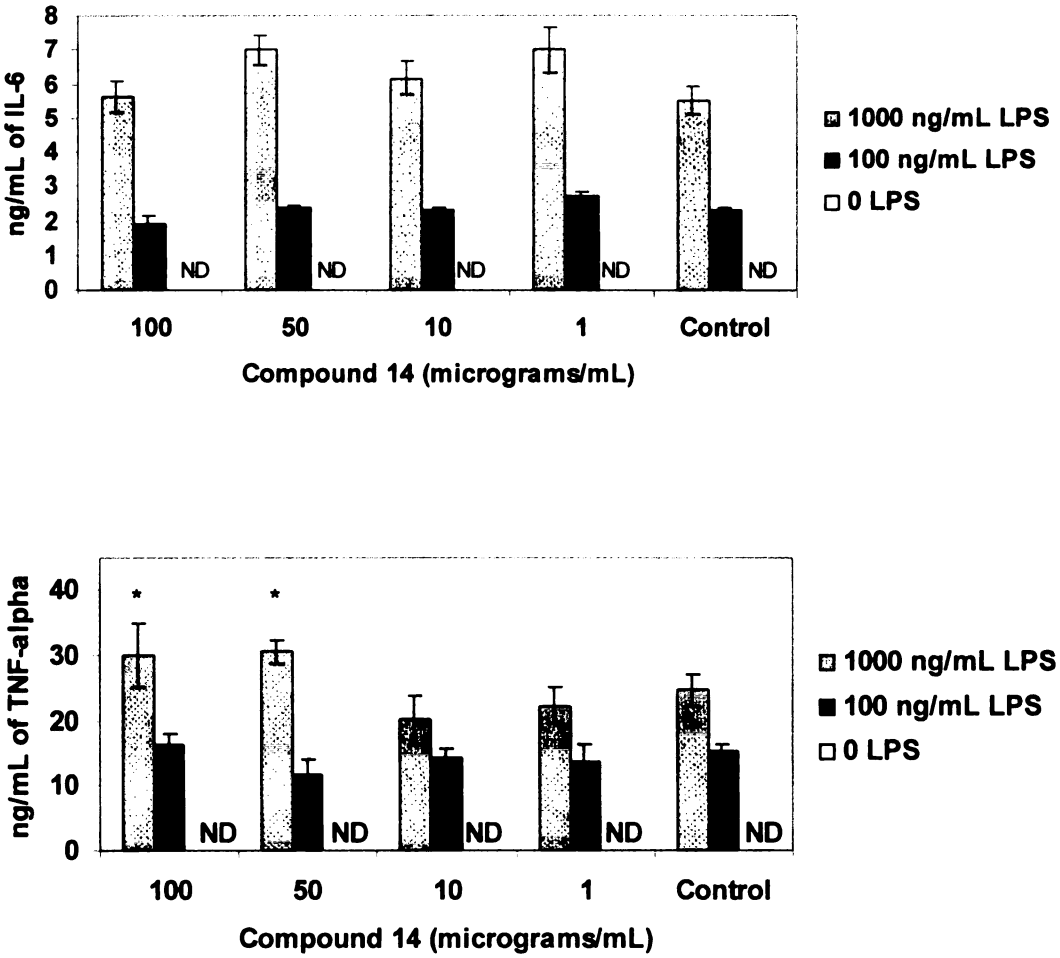


Figure 5.14. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of caftaric acid (**14**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

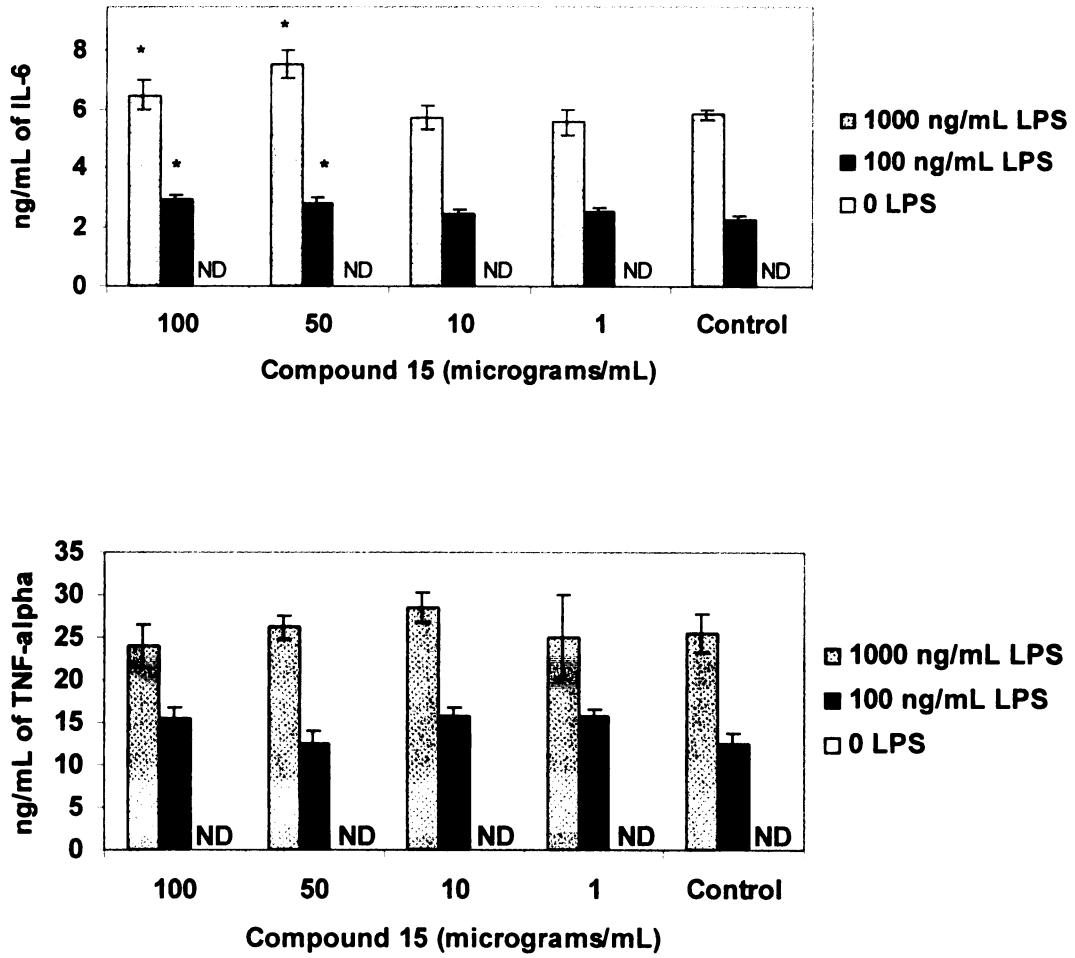


Figure 5.15. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of cichoric acid (**15**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

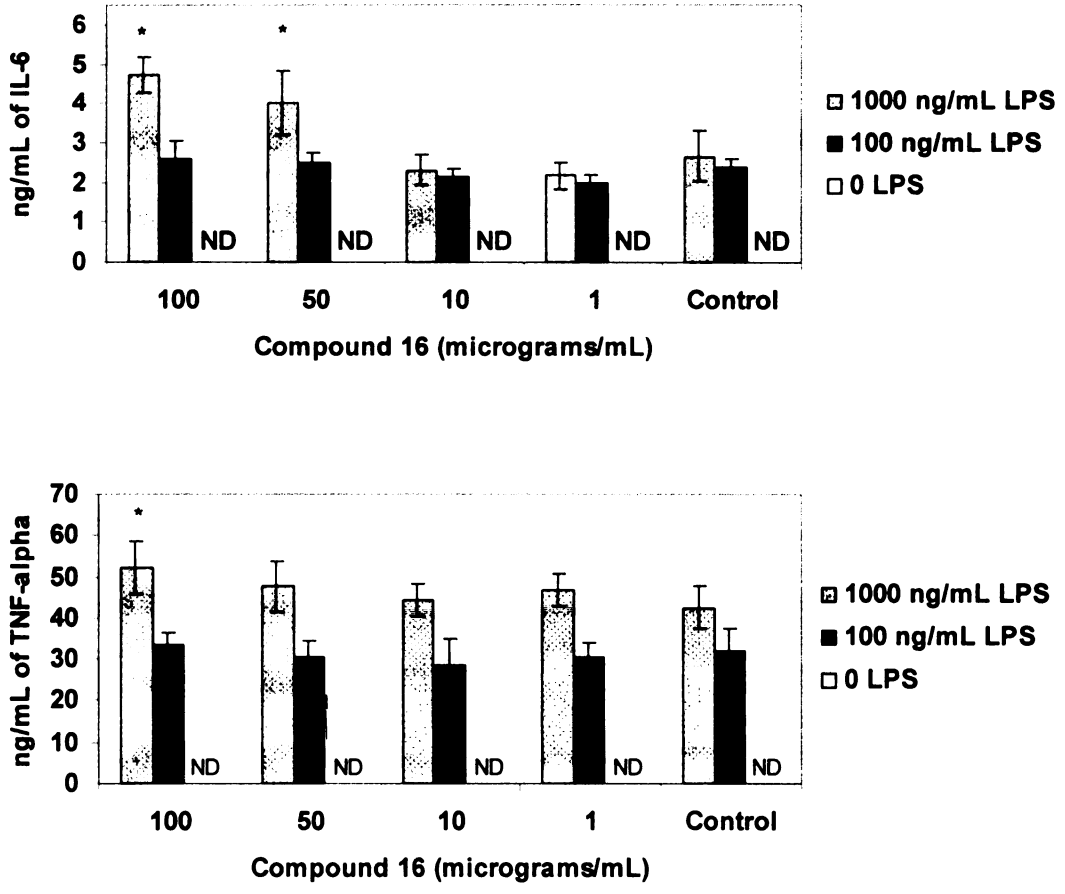


Figure 5.16. Effects of co-treatments of 100, 50, 10, or 1 μg/mL of chlorogenic acid (**16**) with LPS on IL-6 and TNF-α production by RAW 264.7 cells. Asterisks denote treatments (n=3) that significantly differ ($p < 0.05$) from their respective controls (0, 100, or 1000 ng/mL LPS). ND = nondetectable. Results are representative of two independent studies.

stimulating activity. Several compounds did however reduce the levels of IL-6 and TNF-α induced by LPS stimulation of macrophages, with the alkamides most effective at reducing cytokine levels.

Compound **1**, when tested at concentrations of 1, 10, and 50 μg/mL, significantly decreased IL-6 and TNF-α production in cells co-treated with 100 and 1000 ng/mL LPS. When macrophages were stimulated with 100 ng/mL LPS and treated with only 1 μg/mL of compound **1**, significant reductions in IL-6 were noted. TNF-α levels were significantly lower for all doses of compound **1** in cells stimulated with 100 and 1000 ng/mL LPS. Alkamide **2**, structurally similar to compound **1**, showed significant reductions in IL-6 and TNF-α in LPS stimulated cells (**Figure 5.6**). IL-6 levels were reduced following co-treatment of compound **2** at 1, 10, and 50 μg/mL with 100 ng/mL LPS, and at 50 μg/mL for cells treated with 1000 ng/mL LPS. TNF-α was significantly reduced only in cells stimulated with 100 ng/mL LPS and treated with 50 μg/mL of compound **2**.

At 50 μg/mL, the inseparable mixture of compounds **6** and **7** exhibited a significant reduction in IL-6 levels produced by macrophages co-treated with 100 ng/mL LPS. Reduction in IL-6 levels were also observed for compounds **6** and **7** at 1, 10, and 50 μg/mL when macrophages were stimulated with 1000 ng/mL LPS (**Figure 5.8**). TNF-α levels were reduced with 10 and 50 μg/mL of compounds **6/7** in cells stimulated with 100 and 1000 ng/mL LPS, respectively. In contrast to the results obtained for compounds **1**, **2**, and **6/7**, alkamide **4** did not significantly reduce IL-6 or TNF-α levels in LPS stimulated cells (**Figure 5.7**).

The anthocyanins, compounds **8** and **9**, also failed to significantly reduce levels of IL-6 produced following stimulation by 100 or 1000 ng/mL LPS (**Figures 5.9-5.10**). Furthermore, TNF-α levels were not significantly altered by compound **8**; however, compound **9** reduced levels of TNF-α with co-treatments of 100 ng/mL LPS. The flavonoids, compounds **11** and **12**, did not significantly reduce IL-6 levels with stimulation by 100 or 1000 ng/mL LPS (**Figures 5.11-5.12**). Compound **11**,at doses of 10, 50, and 100 μg/mL, reduced TNF-α levels with co-treatments of 100 and 1000 ng/mL LPS. Compound **12** did not affect TNF-α production.

None of the caffeoyl derivatives examined reduced levels of IL-6 in LPS stimulated macrophages (**Figures 5.13-5.16**); however, co-treatments of compound **13** (100 μg/mL), compound **15** (50 and 100 μg/mL), or compound **16** (1, 10, 50, and 100 μg/mL) with 100 or 1000 ng/mL LPS significantly increased levels of IL-6 (**Figures 5.15-5.16**). TNF-α levels were similarly affected, with significant induction noted for compound **13** (50 and 100 μg/mL, 100 and 1000 ng/mL LPS), compound **14** (50 and 100 μg/mL, 1000 ng/mL LPS), and compound **16** (100 μg/mL, 1000 ng/mL LPS) (**Figures 5.13-5.14, Figure 5.16**). No detectable induction of IL-6 or TNF-α occurred without LPS stimulation for compounds 13-16.

Echinacea preparations are generally considered to be safe due to the lack of reported widespread problems associated with their consumption (Parnham, 1996). In previously reported studies, the expressed juice of *E. purpurea* whole plant did not demonstrate in vivo acute or subacute toxicity in

rats or mice, with no relevant differences noted in weight, food consumption, or organ changes upon necropsy (Mullins, 1998). Previous examination of caffeic acid in mouse L-929 cells indicated that cell growth and DNA metabolism were adversely affected only at doses of 500 μg/mL (Cheminat et al., 1988). The alkamides from *E. purpurea* have demonstrated mosquitocidal activity (Clifford et al., 2002); however, the toxicity of these alkamides has not been evaluated in mammalian cell culture assays.

The high dose of alkamides examined in this study, 100 μg/mL, adversely affected RAW 264.7 macrophages based upon the MTT assay results. *Echinacea* alkamides are known to be unstable in raw materials and in root extracts (Kim et al., 2000a; Perry et al., 2000; Perry et al., 1999). This instability should be considered when drawing conclusions regarding the safety of *Echinacea* supplements. Analyses of commercially available *Echinacea* extracts have determined that alkamides are present at very low levels. The cytotoxicity noted with the 100 μg/mL dose in this study indicates that alkamide-containing extracts could pose a safety concern; however, this dose might be too high to be relevant based upon the instability and low concentration of alkamides present in commercially available herbal supplements.

Anecdotal claims indicate that *Echinacea* extracts demonstrate both anti-inflammatory and immune enhancing activities. Reports of headache and fever relief from root extracts suggest an anti-inflammatory effect. Conversely, the use of *Echinacea* to prevent and/or reduce the duration of respiratory tract infections indicates that extracts might cause an inflammatory response. A number of

previously reported studies have supported both anti-inflammatory and inflammatory activities of *Echinacea* extracts.

Adverse reactions noted in the oral administration and injections of *Echinacea* extracts (headache, nausea, fatigue, and tachychardia) (Borchers et al., 2000; Miller, 1998; Jurcic et al., 1989) suggest an inflammatory response in vivo. The cytokine-inducing properties of *Echinacea* extracts and compounds isolated from *Echinacea* have been evaluated to some extent. Treatment of RAW 264.7 murine macrophages with crude *Echinacea* extracts found that *E. purpurea* root and herb materials possessed the ability to activate macrophages to produce IL-6 and TNF-α (Rininger et al., 2000). Previous reports of *E. purpurea* and *E. angustifolia* extracts standardized to 4% phenolic content (chlorogenic acid and cichoric acid) indicated that these extracts did not exhibit macrophage activating activity. Chlorogenic acid, analyzed in addition to the standardized extracts, was not found to induce macrophages to produce IL-6 or TNF-α (Rininger et al., 2000). Quercetin, chlorogenic acid, and caffeic acid have been reported to have no effect on LPS stimulated secretion of IL-6 or TNF-α in human whole blood (Obertreis et al., 1996).

In this study, RAW 264.7 murine macrophage cells were not stimulated by compounds from *E. purpurea* to produce cytokines, as determined by the ELISA quantification of IL-6 and TNF-α. The lack of induction in the absence of LPS stimulation indicates that the alkamides, flavonoids, and anthocyanins did not possess strong immune-stimulating activity. Interestingly, the caffeoyl derivatives demonstrated increased IL-6 and/or TNF-α production when co-treated with 100

or 1000 ng/mL LPS. These results differ from previous reports of crude *Echinacea* extracts, caffeic acid, and chlorogenic acid on cytokine induction. The cell type used in this assay, the duration of exposure to test compounds, and concentrations assayed could have contributed to the opposite results obtained in this study. Caffeic acid (13) administered with 1000 ng/mL LPS afforded levels of TNF-α greater than the other caffeoyl derivatives. While an inflammatory response may not necessarily be beneficial, the ability of caffeic acid to enhance the inflammatory response in this model system could explain the effectiveness of *Echinacea* extracts in the reduction of upper respiratory tract infection severity in clinical trials.

With regard to anti-inflammatory activity of *Echinacea*, alkamides from *E. angustifolia* have previously demonstrated significant in vitro cyclooxygenase-1 inhibitory activity (Müller-Jakic et al., 1994). The ability of *E. purpurea* alkamides to inhibit cyclooxygenase-2 (Clifford et al., 2002), the enzyme inducible upon response to inflammation (Lipsky, 1999), suggests that alkamides might be useful agents in treating inflammation and supports the anecdotal claims of pain and fever relief (Bauer and Wagner, 1991). Caffeic acid has also demonstrated the potential to act as an anti-inflammatory agent. The ability of caffeic acid to prevent the release of IL-6 and TNF-α has been suggested (Bertelli et al., 2002a), and caffeic acid and tyrosol (from white wine), when co-administered with LPS in human peripheral blood mononuclear cells, synergistically inhibited the release of cytokines (Bertelli et al., 2002b).

The alkamides demonstrated the greatest anti-inflammatory activity of the compounds tested, with activity noted at the lowest concentration assayed (1 μg/mL). The results of this assay agree with reported anti-inflammatory in vitro activity of *E. purpurea* and *E. angustifolia* alkamides. With regard to clinical studies, *Echinacea* root extracts are most often administered. Extracts that contain significant concentrations of alkamides might reduce symptoms (headache, fever, pain relief) compared to placebo, resulting in reductions in the severity of symptoms reported by study subjects.

The disparity reported in clinical studies can be ascribed to the chemical composition of the extract used, the species of *Echinacea* from which the extract was prepared, and the plant portion extracted. The chemical profiles of *Echinacea* extracts vary greatly, and extracts that posses greater amounts of anti-inflammatory compounds, such as the alkamides, might reduce or negate any immune-enhancing activity from other compounds present in the extract. Examination of specific constituents from *E. purpurea*, as in this study, more accurately portrays the potential effectiveness of *Echinacea* as an herbal remedy.

Based upon the results obtained in this study, it is conceivable that the compounds present in commercial *Echinacea*-based phytoceuticals may elicit a beneficial anti-inflammatory effect in humans. Alkamides from *E. purpurea* exhibited strong anti-inflammatory activity through the reduction of IL-6 and TNF-α produced. These results support the anecdotal claims of pain and fever relief

associated with *Echinacea* use, and provide evidence for the efficacy of *E. purpurea* as an herbal supplement.

CONCLUSIONS

Echinacea is currently one of the most popular herbal supplements used in the United States. Anecdotal claims of immune enhancement, fever relief, and alleviation of pain have been associated with the use of *E. purpurea* preparations, suggesting that *Echinacea* extracts possess both anti-inflammatory and immune enhancing properties. In order to establish the validity of these anecdotal claims, the efficacy of several compounds isolated from *E. purpurea* was evaluated in antioxidant, anti-inflammatory, and cytokine-induction model systems.

Five primary objectives were defined with regard to the isolation, structure elucidation, and biological testing of active compounds from *E. purpurea* roots and flowers. These objective were 1) isolate and compare COX-1 and COX-2 inhibitory activities of alkamides from *E. purpurea* roots; 2) evaluate the mosquitocidal activity of *E. purpurea* root alkamides; 3) isolate flavonoids and anthocyanins from *E. purpurea* flowers; 4) characterize the COX-1 and COX-2 inhibitory activities and antioxidant activities of the flavonoids and anthocyanins from *E. purpurea* flowers; and 5) characterize and examine the effects of purified constituents from *E. purpurea* roots and flowers (alkamides, flavonoids, and anthocyanins) on cytokine production.

The first objective was accomplished by the isolation and testing of alkamides from *E. purpurea* roots. Seven alkamides, isolated from dichloromethane extracts of *E. purpurea* roots, were examined for

cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activities. Undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide, dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide, and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide were isolated from *E. purpurea* roots. At a concentration of 100 μg/mL, several *E. purpurea* alkamides inhibited COX-1 and COX-2 enzymes in the range of 36-60% and 15-46%, respectively, when compared to controls. The alkamides did not exhibit selective inhibition against either COX-1 or COX-2.

The second objective was to examine other biological activity of *E. purpurea* alkamides. Alkamides, as a class of compounds, are noted for mosquitocidal activity; however, the mosquitocidal activity for alkamides isolated from *E. purpurea* had not been previously established. Several *E. purpurea* alkamides demonstrated mosquitocidal activity at 100 μg/mL, with 100% mortality against *Aedes aegyptii* L. larvae noted. Evaluation of the activity against mosquito larvae established the potential for *E. purpurea* alkamides to be used as mosquitocidal agents.

The biological activity of root extracts from *Echinacea* species have been investigated; however, the flowers have not been fully evaluated. In order to identify bioactive constituents from this underutilized part of *E. purpurea*, the third objective was to isolate and identify constituents from *E. purpurea* flowers for later analysis of their biological bioactive properties. This study led to the

extraction, isolation, and structure elucidation of three flavonoids (quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside) and two anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside) from methanolic extracts of lyophilized *E. purpurea* flowers.

Flavonoids (quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-robinobioside) and anthocyanins (cyanidin-3-*O*-β-D glucopyranoside and cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside), isolated from methanolic extracts of lyophilized *E. purpurea* flowers, and caffeoyl acid derivatives, caffeic acid, caftaric acid, chlorogenic acid, and cichoric acid, were examined for antioxidant and COX-1 and COX-2 inhibitory activities. The anthocyanins demonstrated the greatest cyclooxygenase inhibitory activities at 100 μg/mL, with inhibition of COX-1 and COX-2 ranging from 26-28% and 23-26%, respectively. When assayed at a concentration of 10 μg/mL, the antioxidant activity of these compounds ranged from 33-81% for the anthocyanins, 3-54% for the flavonoids, and 35-79% for the caffeoyl derivatives.

The fourth objective was to examine the cyclooxygenase inhibitory and antioxidant activities of compounds from *E. purpurea* flowers. Currently, *E. purpurea* flowers are included in extracts of the fresh herb only if the flower is in bloom when the above-ground portion of the plant is harvested. The promising results obtained in this study indicate that extracts prepared from *E. purpurea* flowers might provide a concentrated source of bioactive compounds, and further

studies regarding the development of an *E. purpurea* flower-based natural product supplement is warranted.

Previous evaluation of the effects of crude *E. purpurea* extracts on LPS-stimulated RAW 264.7 murine macrophages revealed their ability to induce cytokines to produce IL-6 and TNF-α. The fifth objective was to determine the effects of cytokine production in murine macrophage cells (RAW 264.7) co-treated with compounds isolated from *E. purpurea* and lipopolysaccharide (LPS), an inflammagenic stimulus. An assessment of the cytotoxicity of the doses tested in the macrophage assay determined that the highest concentration of alkamides used, 100 μg/mL, adversely affected cells.

No induction of IL-6 or TNF-α occurred without LPS stimulation in macrophages treated with test compounds. The alkamides examined in the assay were highly effective at reducing LPS-stimulated cytokine induction when compared to controls. The anti-inflammatory activity of alkamides in the macrophage assay supports the anecdotal claims of pain and fever relief associated with the use of *Echinacea* roots, from which the alkamides were isolated.

An increase was noted in IL-6 and TNF-α production when caffeoyl derivatives were co-treated with LPS, suggesting the potential for immune-enhancing properties of *Echinacea* extracts. These results demonstrate that individual compounds from *E. purpurea* might possess opposing activities with regard to immune stimulation and/or suppression. This might explain why the

immune-enhancing activity of *Echinacea* extracts as reported in the literature varies widely.

The results of the fifth objective indicate that examining single constituents from *Echinacea* extracts, rather than crude extracts, provides more detailed information regarding cytokine induction and/or suppression in LPS-stimulated macrophages. The alkamides demonstrated striking anti-inflammatory activity in the model system, while conversely, the caffeoyl derivatives in combination with LPS resulted in the induction of cytokines. The effectiveness of crude preparations of *Echinacea* will vary depending upon the qualitative and quantitative chemical profile of the extract.

Alkamides, flavonoids, anthocyanins, and caffeoyl derivatives from *E. purpurea* demonstrated antioxidant, cyclooxygenase-inhibitory, mosquitocidal and cytokine induction and/or suppression activities. *Echinacea* supplements are already among the most popular herbal supplements on the market, and these findings help give credence to their continued use. Further studies are needed in order to further define the bioactivity profiles of *Echinacea*-derived constituents. Ultimately, this research will contribute to the development of new *Echinacea*-based phytoceuticals tailored to provide consistent, safe, and effective pharmacological activities.

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APPENDIX A. CONVERSION OF ASSAY CONCENTRATIONS.

#	Compound	100 μg/mL	50 μg/mL
1	Undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide	437 μM	218 μM
2	Undeca-2*Z*,4*E*-dien-8,10-diynoic acid isobutylamide	437 μM	218 μM
3	Dodeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide	412 μM	206 μM
4	Undeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide	412 μM	206 μM
5	Dodeca-2*E*,4*Z*-dien-8,10-diynoic acid 2-methylbutylamide	389 μM	195 μM
6/7	Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide / dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	405 μM	202 μM
8	Cyanidin-3-*O*-β-D-glucopyranoside	223 μM	111 μM
9	Cyanidin-3-*O*-malonyl-(1→6)-β-D glucopyranoside	187 μM	93.5 μM
10	Kaempferol-3-*O*-robinobioside	168 μM	84 μM
11	Quercetin-3-*O*-rutinoside	164 μM	82 μM
12	Quercetin-3-*O*-glucoside	216 μM	108 μM
13	Caffeic acid	556 μM	278 μM
14	Caftaric acid	320 μM	160 μM
15	Cichoric acid	211 μM	106 μM
16	Chlorogenic acid	282 μM	141 μM