ECHINACEA AS A FUNCTIONAL FOOD INGREDIENT

CLIFFORD HALL III

324 IACC, Department of Cereal and Food Sciences
North Dakota State University
Fargo, ND 58105
USA

I. INTRODUCTION

Echinacea (pronounced ek-a-NAY-sha) or purple coneflower is a perennial plant in the Compositae or daisy family (Foster, 1985). Traditional literature has reported that nine species of *Echinacea* exists in nature. However, under a new reclassification system, eight varieties are categorized under only four species (Binns *et al.*, 2002a). *Echinacea* (*E.*) *pallida* var. *angustifolia* (hereafter referred to as *E. angustifolia*) and *E. purpurea* are the most common species of *Echinacea*. *E. angustifolia* is a wild flower in North

ADVANCES IN FOOD AND NUTRITION RESEARCH VOL 47
ISSN: 1043-4526
DOI: 10.1016/S1043-4526(03)47003-4

Dakota and other parts of the North American great plains that range from Texas to Saskatchewan whereas *E. purpurea* can be found from Georgia to Michigan and into Kansas in the west (Foster, 1991). The flower size and color vary among species of *Echinacea* but in general they can be characterized by hues of purple ray florets that surround an orange-brown colored head containing numerous achene, i.e., fruiting bodies that contain seeds (Foster, 1985; Schulthess *et al.*, 1991). *E. purpurea* (L.) Moench., *E. angustifolia* DC, and *E. pallida* var. *pallida* Nutt. (hereafter referred to as *E. pallida*) are the *Echinacea* species most widely used for medicinal purposes and are commercially cultivated. In the western United States and Canada, *E. purpurea* (L) Moench. and *E. angustifolia* DC. account for 80 and 20%, respectively, of the cultivated *Echinacea* (Li, 1998). Other species (*E. laevigata*, *E. atrorubens*) and varieties (*E. tennesseensis*, *E. sanguinea*, *E. simulata*, and *E. paradox*) are adapted to specific growing regions and are thus not exploited commercially.

In 1909, the American Medical Association dropped *Echinacea* from the list of approved medicinal agents (Wills *et al.*, 2000). In contrast, the European Community embraced *Echinacea* as a pharmaceutical agent. By 1930, German researchers began exploring the chemical constituents of *Echinacea* in the hope of identifying the component(s) responsible for the biological activity (Wills *et al.*, 2000). Over the past 70 years, a number of components have been identified as having biological activity. It is widely accepted today that the active constituents are grouped into the unsaturated lipophilic compounds (Figure 1), caffeic acid phenols (Figure 2), and polysaccharides (Figure 3) categories. However, synergistic activities may exist with other components of *Echinacea* to provide bioactivity.

Around the turn of the 20th century, *Echinacea* was being prescribed as a treatment for a number of aliments, which included snake bites, typhus, dysentery and cancer (Wills *et al.*, 2000). Today, *Echinacea* is promoted as an immunostimulatory agent (Bauer, 1999a, 2000) and is one of the most popular dietary supplements in the United States. In addition, *Echinacea* has been used successfully in Germany as evidenced by the two million prescriptions filled by German physicians annually (Barrett *et al.*, 1999). Supplements or preparations of *Echinacea* are derived from the herbal (including seeds or flowers) and root or rhizome parts of the plant. Numerous preparations exist in the market, which include fresh and dried plant materials, expressed juices, ethanol tinctures and glycerin extracts.

Most of the available *Echinacea* preparations could be incorporated into food systems to create a functional food. Teas, beverages, and confections, for example, have been targeted as delivery agents of *Echinacea* phytochemicals in food systems (Wills *et al.*, 2000). The use of *Echinacea* as a component in functional foods has come under fire because *Echinacea* is not considered

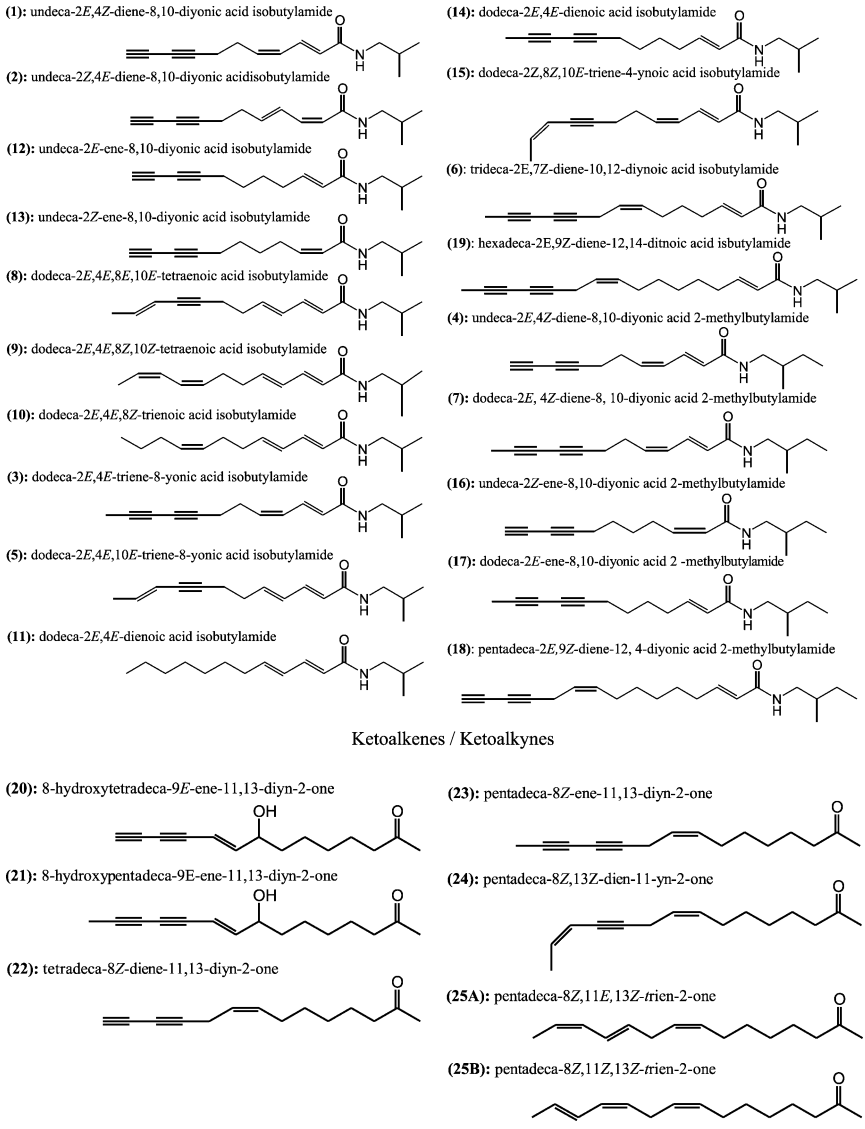

FIG. 1 Alkamides and ketoalkenes/alkynes identified in *Echinacea*. Numbers in parentheses refer to the numbers assigned by Bauer and Remiger (1989).

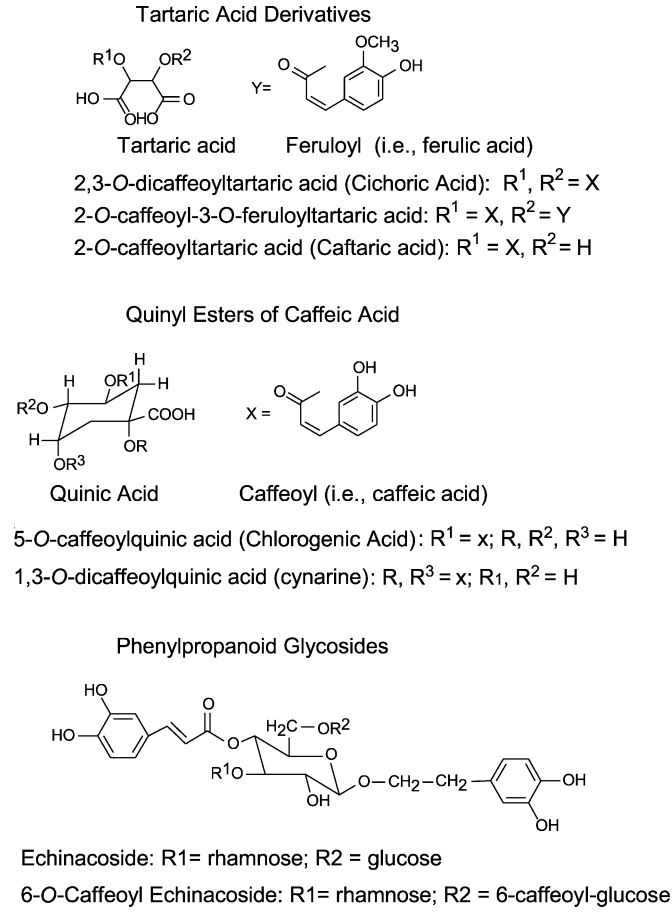

FIG. 2 Major caffeic acid phenols (CAP) found in *Echinacea*.

a "generally recognized as safe" (GRAS) ingredient in the United States. However, Health Canada does support the use of *Echinacea* in food products and functional foods could be developed for the Canadian market (Health Canada, 1999). The economic importance of *Echinacea* in the dietary and medicinal markets is significant, and thorough studies need to be completed to assess the risk/benefits of consuming *Echinacea*. In addition, phytochemical stability assessments are critical if *Echinacea* is to be incorporated into food systems. This chapter will highlight the phytochemical constituents of *Echinacea* and will include information relevant to the biological activity,

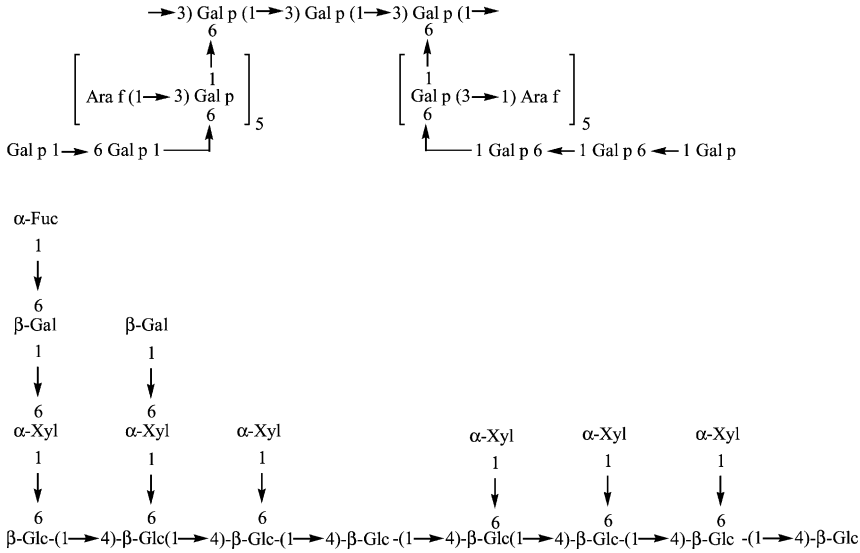

FIG. 3 Example of polysaccharide obtained from *Echinacea* and cell cultures of *Echinacea*. Partially characterized structures (adapted from Wagner *et al.*, 1988; Melchart and Linde, 1999).

potential safety concerns, detection methods and the effects of processing on phytochemicals, including potential functional activities such as antioxidant and antimicrobial activities. Limited information will be provided for growing and harvesting of *Echinacea*. It is the author's goal to present the most current information; thus a great number of studies may not be presented in which the author suggests that the reader seek for additional background. In addition, a variety of studies showing both positive and negative results have been presented in the hope that a more standard method be adopted for assessing the biological activity of *Echinacea*.

II. PHYTOCHEMICAL CONSTITUENTS

A. GENERAL

Unsaturated lipophilic compounds, caffeic acid phenols (CAP) and polysaccharides are believed to be responsible for the observed immunostimulatory activity. The alkamides and ketoalkenes/alkynes (Figure 1) are the two major groups in the unsaturated lipophilic compound category.

Approximately, 20 alkamides and seven ketoalkenes/alkynes have been characterized (Bohlmann and Hoffman, 1983; Bauer *et al.*, 1987; Bauer and Remiger, 1989; He *et al.*, 1998; Dietz and Bauer, 2001). Twelve (12) CAP (Figure 2) have been characterized and classified as quinyl esters of caffeic acid, tartaric acid derivatives, or phenylpropanoid glycosides (Becker *et al.*, 1982; Becker and Hsieh, 1985; Bauer *et al.*, 1988a,b; Cheminat *et al.*, 1988). In addition, at least eight phenolic acids have been identified (Glowniak *et al.*, 1996). The polysaccharides are the least characterized of the three main categories; however, approximately five polysaccharides and three glycoproteins have been characterized (Proksch and Wagner, 1987; Wagner *et al.*, 1988; Roesler *et al.*, 1991a; Bauer, 1999a; Classen *et al.*, 2000). Other components such as flavonoids and essential oils are present in various parts of the plant and could act to enhance the biological activity of *Echinacea*.

B. LIPOPHILIC COMPOUNDS

1. Concentrations and composition

The immune-enhancing activity of the lipophilic compounds is attributed to the alkamides. Isobutylamides and 2-methylbutylamides are the two general classes of alkamides (Bauer and Remiger, 1989) based on the isobutyl or methylbutyl moiety linked via an amide bond to an unsaturated hydrocarbon (Figure 1). The ketoalkenes/alkynes lack the butylamide moiety and could be artifacts formed during storage (Bauer *et al.*, 1987). The most common methods for the extraction of lipophilic constituents include the use of hexane (Bauer *et al.*, 1989; He *et al.*, 1998) or acetonitrile (Perry *et al.*, 1997). Most researchers use HPLC to separate the alkamides. Typical conditions include a reverse-phase column and a mobile phase of water and acetonitrile (40–80%, gradient) and detection at 254 nm (Bauer *et al.*, 1988c, 1989).

Total alkamide levels in *Echinacea* are variable and limited data are available for direct comparisons between laboratories (Table I). However, most researchers agree that the roots contain higher alkamide levels than the aerial parts (Bauer and Remiger, 1989; Perry *et al.*, 1997; Wills and Stuart, 1999; Stuart and Wills, 2000a,b; Binns *et al.*, 2002b). Stuart and Wills (2000a) reported that approximately 70% of the alkamides were found in the roots followed by the flower (20%), stem (10%) and leaves (1%). Further dissection of the *Echinacea* plant gave similar alkamide distributions with 24% of the alkamides being obtained from the reproductive stem, followed by the roots (22%), flowers (19%), rhizomes (17%), vegetative stems (14%) and leaves (4%) (Perry *et al.*, 1997).

Wills and Stuart (1999) and Stuart and Wills (2000a) completed a series of experiments to evaluate alkamide levels in *E. purpurea* grown in Australia.

TABLE I
AVERAGE ALKAMIDE CONTENT (mg/g PLANT PART DRY WEIGHT) OF VARIOUS PLANT PARTS OF *E. PURPUREA*

	Plant part					
Location	Roots	Rhizome	Flower	Leaf	Veg. stem[a]	Rep. stem[b]
Australia[c]	8.9	n/r	2.6	0.10	n/r	0.75
Australia[d]	3.9[e]	n/r	0.66[f]	–[f]	–[e]	–[f]
New Zealand[g]	6.2	8.0	2.9	0.24	19.0	1.52
United States[h]	12.9	n/r	5.63[i]	n/r	n/r	–[i]

[a]Vegetative stem.
[b]Reproductive stem; n/r = not reported.
[c]Wills and Stuart (1999).
[d]Rogers *et al.* (1998).
[e]Includes vegetative stems.
[f]Reported as aerial parts but may include flower, leaf and reproductive stem parts.
[g]Perry *et al.* (1997).
[h]Binns *et al.* (2002a).
[i]Reported as inflorescences (flower and reproductive stem parts).

Of the 62 commercial samples tested, 50% of the samples had alkamide levels in the range 6–9 mg/g roots (d.w.b.) and 35% fell in the range 3–6 mg alkamide/g root material. In addition, 90% of the samples derived from the aerial parts (leaves, stems, flowers) had alkamide levels in the range 0.2–1.4 mg/g dried aerial parts (Wills and Stuart, 1999). This same study showed that alkamide levels were higher in samples obtained in the areas north of the 32°S latitude in Australia. Further studies showed that coastal (32°S latitude, sea level, 13–23°C) growing sites had slightly higher, but not significant, levels of alkamides than the sub-tropic tableland (32°S latitude, 1030 m altitude, 13–23°C) during the first growing season (Stuart and Wills, 2000a). However, the level of alkamides in the tableland flower tissue collected during the second season, and at the mature growth stage, was significantly higher than the alkamide levels from flower tissue of coastal *E. purpurea*. Stuart and Wills (2000a) also found that alkamide levels decreased as the tissue aged during the growing season. For example, the concentration of alkamides dropped from 11.7 mg/g root tissue, collected at the pre-flowering stage, to 10.2, 9.5, and 9.0 mg/g during the flowering, mature and senescent growth stages, respectively. However, an overall accumulation of alkamides was found per plant due to an increase in biomass during the growing season.

Rogers *et al.* (1998) reported alkamide levels in *E. purpurea* between 0.24 and 1.1 mg/g aerial parts for samples collected at various locations within Australia. Again, the growing location did not have a significant effect

on alkamide formation. These authors also noted that the vegetative stem and root portions gave higher alkamide levels (3.9 mg/g), which is in agreement with the other researchers. The average alkamide levels in the root of *E. angustifolia* grown in the United States and Australia were 1.2 and 0.59 mg/g root, respectively (Rogers *et al.*, 1998). However, only a few samples were evaluated in this study and in one Australian sample the alkamide level of 1.1 mg/g root was found, again indicating that the growing location may not significantly influence alkamide concentrations.

Binns *et al.* (2002b) evaluated the alkamide levels in cultivated and wild populations of various *Echinacea* species and varieties. In most samples, the concentration of alkamides was highest in the root material. However, no clear trend was observed across all species and varieties under all growing conditions. Only the flowering parts of *E. pallida* obtained from plants grown from wild-harvested seeds in the greenhouse (referred to as germlings) had alkamide levels higher than the root material of the same plant. With the exceptions of *E. purpurea* and *pallida*, the roots of the germlings had total alkamide levels higher than wild-harvested roots (Table II). Transplanting of the wild species/varieties and growing under greenhouse conditions generally favored the production of alkamides (Binns *et al.*, 2002b). The most interesting samples were that of *E. atrorubens* var. *atrorubens*. The cultivated samples had significantly higher alkamide levels than the wild-harvested samples. For example, the cultivated samples had alkamide levels of 59.5 and 22.6 mg/g root tissue for the germlings and transplants, respectively; whereas, the wild-harvested had only 4.9 mg alkamides/g root tissue (Table II). For a general summary of the total alkamides in the roots of *Echinacea*, please see Table II and for additional details please refer to the report by Binns *et al.* (2002b). In addition, wild-harvested

TABLE II

AVERAGE TOTAL ALKAMIDE[a] CONTENT (mg/g DRY ROOT) OF VARIOUS *ECHINACEA* SPECIES AND VARIETIES GROWN UNDER CULTIVATED AND WILD ENVIRONMENTS

	Germlings[b]	Wild-harvested	Wild-transplant
E. purpurea	12.9	14.2	17.4
E. pallida var *angustifolia*	11.1	10.7	24.1
E. pallida var *pallida*	1.3	8.6	2.3
E. pallida var *sanguinea*	53.1	36.1	14.3
E. pallida var *tennesseensis*	28.6	14.2	16.2
E. atrorubens var *atrorubens*	59.5	4.9	22.6

Adapted from Binns *et al.* (2002a).

[a]Estimated average total alkamide levels.

[b]Plants grown from wild-harvested seeds in a greenhouse.

E. atrorubens var. *paradox* and *E. atrorubens* var. *neglecta* had significantly higher levels of ketoalkenes/alkynes than other wild-harvested *Echinacea* (Binns *et al.*, 2002b). Cultivating the *E. atrorubens* var. *neglecta* in the greenhouse had little effect on the production of the ketoalkenes/alkynes, as noted by the slight reduction in root alkamide levels from 6.7 to 4.3 mg/g in wild-harvested and cultivated samples, respectively. In contrast, cultivating of *E. pallida* slightly promoted the formation of ketoalkenes/alkynes from 1.0 in wild-harvested to 1.9 and 3.3 mg/g root tissue for transplants and germlings, respectively. For most other *Echinacea* species/varieties, the cultivated samples had lower ketoalkenes/alkynes levels than the wild-harvested plants. Similar to the alkamide, the root tissue had higher concentrations of the ketoalkenes/alkynes than did the flower tissue (Binns *et al.*, 2002b).

The concentration of individual alkamides appeared to depend more on the tissue type than on the growing location and conditions. Twenty (20) alkamides have been identified in *Echinacea* and not one species/variety contains all 20 alkamides (Bohlmann and Hoffman, 1983; Bauer *et al.*, 1987; Bauer and Remiger, 1989; He *et al.*, 1998; Dietz and Bauer, 2001). In general, alkamides in *E. purpurea* possess a 2,4-dienoic moiety, whereas the 2-monene moiety is more common in *E. angustifolia*. The 2-ketoalkenes and 2-alkynes lack an isobutylamide moiety and are common for *E. pallida* (Bauer *et al.*, 1988c; Bauer and Remiger, 1989; Schulthess *et al.*, 1991). Regardless of species/variety, the roots appear to have a more diverse alkamide profile than the herbal/aerial parts. However, dodeca-2*E*,4*E*,8*Z*, 10*Z*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide (hereafter referred to as the tetraenoic alkamides) are the major alkamides in *Echinacea*.

Bauer and Remiger (1989) reported that the tetraenoic alkamides content was 0.04–0.39 mg/g root of *E. purpurea* and 0.09–1.51 mg/g root of *E. angustifolia*. The aerial parts had less than 0.01–0.3 mg/g of these alkamides in samples obtained from the United States and Europe. Rogers *et al.* (1998) found similar levels of tetraenoic alkamides in Australian-grown *E. purpurea* and *E. angustifolia*. Perry *et al.* (1997) observed that the tetraenoic alkamides accounted for 27% of the alkamides in the roots, 71% in the rhizome and 74% in the vegetative stem. These three parts are often harvested as a root and thus collectively the root system accounted for 84% of the total tetraenoic alkamides in the *E. purpurea* plant tissue. In the aerial parts of the plant, 64% of tetraenoic alkamides were found in the flower, and 31 and 5% were found in the reproductive stem and leaves, respectively. In *E. purpurea*, the tetraenoic alkamides accounted for 45 and 76% of the alkamides from the roots and aerial parts, respectively (Wills and Stuart, 1999). Additional research showed that the tetraenoic alkamides dodeca-2*E*, 4*E*, 8*Z*, 10*Z*-tetraenoic acid isobutylamide and

dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide accounted for 13 and 32% of the total alkamides in the root tissue (or 29 and 71% of tetraenoic alkamides), respectively (Stuart and Wills, 2000a). Binns *et al.* (2002b) also reported that the tetraenoic alkamides were the predominant alkamides in all *Echinacea* species/varieties except *E. pallida* var. *tennesseensis*. In this particular variety, undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide was the major (45%) alkamide in the root tissue. However, the tetraenoic alkamides were the predominant alkamides in the aerial parts in all the species/varieties. With a few exceptions, the alkamide distribution was similar between the cultivated and wild-harvested plants (Binns *et al.*, 2002b).

Other predominant alkamides include undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (Wills and Stuart, 1999; Stuart and Wills, 2000a; Binns *et al.*, 2002b). In *E. purpurea*, these alkamides accounted for 15–25 and 13–27% of the root alkamides, respectively. However, neither compound was found in the aerial parts (Wills and Stuart, 1999; Stuart and Wills, 2000a; Binns *et al.*, 2002b). Undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide accounted for 20% of the alkamides in the aerial parts and only 6% in the root of *E. purpurea* (Wills and Stuart, 1999). This observation was further supported by Binns *et al.* (2002b) who found that undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide was predominantly present in the aerial parts for most species/varieties tested. These authors found that this alkamide accounted for 29% of the total alkamides from the aerial parts of *E. atrorubens* var. *atrorubens*, which was the highest average percentage among the plants tested. Additionally, they found that this alkamide was more prevalent in the cultivated plants and that most wild *Echinacea* plants tested lacked this alkamide (Binns *et al.*, 2002b). The cultivation of *Echinacea* is widely practiced and the higher alkamide levels found in cultivated plants provide sufficient evidence to eliminate the wild-harvesting practices.

2. Alkamide isolation and key structural features

Although a great number of advances have been made in the understanding of *Echinacea*, only limited information is available on the health benefits, chemical interaction, and safety of individual components or mixtures of components. From a practical perspective, using single components has little significance considering the make-up of *Echinacea* preparations currently used for pharmaceutical purposes. However, from a basic scientific perspective many additional questions are still not fully answered, such as the bioavailability and characterization of potential degradation products. Information on the isolation and characterization of components for basic studies is provided below. Additional information on how the components

are affected by processing/extraction of *Echinacea* will be discussed in later sections.

Two of the most common methods for the extraction of alkamides involve the use of hexane (Bauer *et al.*, 1989; He *et al.*, 1998) or acetonitrile (Perry *et al.*, 1997); however, ethanol-based extractions have also proven successful (Livesey *et al.*, 1999; Stuart and Wills, 2000b). In our laboratory at North Dakota State University, we use a series of solvents starting with hexane, then ethanol and finally 70% ethanol to extract the various families of compounds (e.g., lipophilic). Regardless of the extraction method, separation of individual alkamides can be completed using the thin layer chromatography (TLC) method of Bauer and Remiger (1989). Typically, hexane extracts are yellow and can be fractionated over silica gel using ethyl acetate–hexane (1:5) or by TLC using ethyl acetate–hexane (1:2). Bauer and Remiger (1989) used silica gel plates containing fluorescence indicator (F_{254}) and anisaldehyde/sulfuric acid spray, for detection, as a screening method on fractions collected during column chromatography. They found that the 2-monoenamide structures gave a yellow color while a violet color was formed when 2,4-dienamides were present. The 2,4-dienamides fluoresce at 254 nm, thus the plate can be viewed under UV light and used as a method for preliminary identification. Further purification of the alkamides can be completed on a reverse-phase semi-preparative column using water and acetonitrile (40–80%) gradient and detected at 254 nm (Bauer *et al.*, 1988c, 1989). When separating natural products, confirmation of the structural features is needed if an authentic source is not readily available. Nuclear magnetic resonance (NMR) data for the alkamides are similar because most contain an isobutylamide moiety and an unsaturated carbon chain. The variation in NMR data reflects the position and type of chemical bonds present in the alkamide.

The isobutylamide region of the alkamides (Figure 1) is unique and can be used to characterize the alkamides from the 2-methyl alkamides and other olefins. The broad singlet at *ca.* 5.5 ppm represents the proton on the nitrogen (N–H) whereas the doublet at 0.93 ppm represents the methyl protons ($2CH_3$). The methylene proton next to the nitrogen ($-NH-CH_2-$) is represented by a double doublet at 3.18 ppm and the multiplet signal at 1.80 ppm represents the methine protons ($-NH-CH_2-CH-$). If the isobutylamide feature were replaced by a 2-methyl-butylamide substitution (Figure 1), the most obvious change would be the additional triplet signal at 0.91 ppm, which represents the protons of the methyl group adjacent to the methylene group. The presence of a multiplet signal at 1.41 and 1.16 ppm represents the methylene protons adjacent to the methyl and methine groups. The proton signal at 1.58 ppm represents the methine group next to nitrogen.

The unsaturated carbon moiety will produce signals in two primary regions. The signals between 5.87 and 7.51 ppm represent the olefin protons

while the allylic methylene protons resonate at 2.18 and 2.55 ppm. A proton on terminal acetylene (i.e., triple bond) would have a signal near 1.98 ppm. Coupling constants of 11 and 15 Hz correspond to the *Z* and *E* configurations, hand are thus important for establishing the stereochemistry of the alkamide. For additional discussion on proton NMR for the alkamides, see Bauer *et al.* (1988c, 1989). Perry *et al.* (1997) provided ^{13}C NMR data that showed a characteristic carbonyl carbon at 166 ppm, which corresponds to the carbon attached to the nitrogen via an amide bond. The aliphatic carbon signals can be found between 11 and 47 ppm whereas carbon signals associated with the double and triple bonds of the unsaturated chain can be found between 119–136 and 64–77 ppm, respectively.

Mass spectral data is a useful analytical tool for characterizing structural features of the alkamides. Typically, the mass to charge (*m*/*z*) ratios ranged from 229 to 278 for the alkamides (He *et al.*, 1998). The isobutylamides and 2-methyl-butylamides can be differentiated by evaluating the fragmentation patterns. Isobutylamides include $[M - 57]^+$, $[M - 72]^+$, and $[M - 100]^+$, while 2-methyl-butylamides have $[M - 29]^+$, $[M - 86]^+$, and $[M - 114]^+$. The loss of 57, 72 and 100 mass units indicates a loss of the isobutyl group $[C_4H_9]^+$, $[C_4H_{10}N]^+$ and $[C_4H_{10}NCO]^+$, respectively. The loss of 29 mass units from 2-methyl-butylamides represents a $[C_2H_5]^+$ or ethyl ($-CH_2-CH_3$) unit. The loss of $[C_5H_{12}N]^+$ and $[C_5H_{12}NCO]^+$ are indicated by a loss of 86 and 144 *m*/*z* units from the parent ion.

3. Role of processing on alkamide recovery and stability

Because of the unsaturated nature of the alkamides, the oxidative stability of the alkamides may be a potential concern during the storage and processing of *Echinacea*. Bauer *et al.* (1988b) found a reduced alkamide level in stored *E. pallida*. They also noted that the ketoalkenes/alkynes undergo oxidative reactions, particularly in chopped samples. The storage of herbal medicines containing *Echinacea*, in dry form, would be expected to have similar alkamide reductions. Thus, reducing the potential for oxidation during storage and processing is critical for maintaining optimal alkamide levels in *Echinacea*-containing products.

Alkamide concentrations increased when *Echinacea* was subjected to various physical treatments that included cutting, compression or crushing (Wills and Stuart, 2000). The alkamide concentration increased from 6.0 mg/g in undamaged plant tissue to 9.6 mg/g in cut plant tissue. Bruising of the tissue prior to drying did not enhance alkamide retention; however, bruising followed by cutting significantly enhanced alkamide retention to about 9.2 mg/g (Wills and Stuart, 2000). These authors hypothesized that the higher alkamide concentrations observed in the physically abused samples

were due to the faster drying time as opposed to a biosynthetic production of alkamides. The physically abused samples were dried to less than 12% moisture in 12 h at 40°C whereas the whole plant required 48 h to reach the same moisture level. Kabganian *et al.* (2002) observed that the drying rate had two phases in which the moisture reduction was initially slow, but increased rapidly when the moisture content of the plant material was approximately 35%. These authors attributed the increase in drying rate to a cell-disruption phenomenon such that during the initial drying process a molecular diffusion across membranes was the mechanism for dehydration. The second phase of drying was attributed to a convective mass transfer where the cells disrupt allowing for the release and evaporation of water more quickly, thus increasing the drying rate. For example, drying *Echinacea* from 57 to 10% moisture at 30°C required 103.4 h whereas 5.3 h was required for drying at 70°C.

In addition, Kabganian *et al.* (2002) reported that the alkamide levels were not significantly affected by the drying process, which supports the findings of Wills and Stuart (2000) who found that the more rapid the drying time, the greater the alkamide retention. Total alkamide levels of *E. purpurea* roots were not significantly affected by drying (32°C for 48 h) from 69.2 to 6.8% moisture (Perry *et al.*, 2000). However, a significant reduction in the concentrations of two alkamides was found in the chopped *E. purpurea* roots. Dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamides and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide levels each dropped by 13% simply by chopping the root and rhizome tissue. A greater reduction (16 and 23%, respectively) was found after the samples had been chopped and then dried for 48 h.

The storage length and temperature are critical factors in the oxidative stability of polyunsaturated compounds. This also held true for the alkamides. Bauer *et al.* (1988b) noted that 8-hydroxy-9-ene derivatives were formed during the storage of *E. pallida*. They noted that the ketoalkenes/alkynes undergo oxidative reactions and suggested that the root remains whole and that the extract remains in solution to prevent oxidative degradation. Rogers *et al.* (1998) found that the storage of powdered *E. angustifolia* roots in a sealed bag at room temperature, under desiccation, resulted in a reduction in alkamide levels by 13%. A loss of 40, 55 and 80% in alkamide levels were found in the chopped *E. purpurea* samples stored at −18, 3 or 24°C respectively for 64 weeks, (Figure 4; Perry *et al.*, 2000). Livesey *et al.* (1999) reported a significant loss in the alkamide levels in powdered *E. purpurea* roots as temperatures increased from −20 to 40°C. These authors reported alkamide levels of 7.5 mg/g root for powders stored at −20°C; whereas, alkamide levels of 1.1 and 0.2 mg/g root were found in samples stored at 25 and 40°C, respectively. In contrast, no significant reductions of alkamides

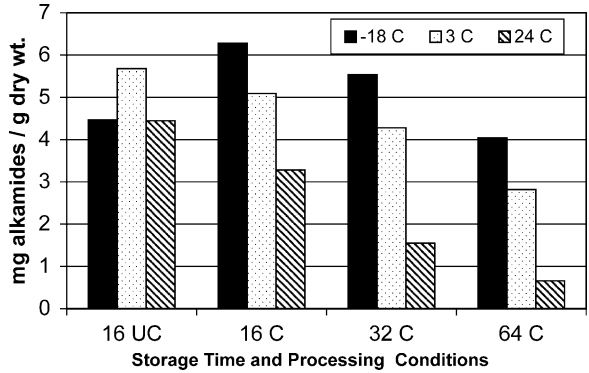

FIG. 4 The effect of storage time (days) and processing (chopped (C) or unchopped (UC)) conditions on alkamide retention (adapted from Perry *et al.*, 2000).

were found when alkamides were stored under acetonitrile (Perry *et al.*, 2000) or methanol (He *et al.*, 1998), which is further supported by the observation that alkamide stored under dry conditions (i.e., free of solvent) were unstable (Jacobson, 1954). Although acetonitrile would not be used in tinctures, the alkamides may behave in a similar manner under ethanol-preserved tinctures as demonstrated by Stuart and Wills (2000b). No significant change in alkamide levels were found in ethanol (55%) extracts stored at temperatures between −20 and 40°C (Livesey *et al.*, 1999).

Alkamide concentration increased during storage of *E. purpurea* roots at 20°C and 60% relative humidity (RH) over a 20-day storage period (Wills and Stuart, 2000). However, a loss, although not significant, of alkamides was reported after an additional 10-day storage. Storage of the aerial parts under the same conditions did not affect the alkamide retention over the 30-day storage. In contrast, 70 and 55% reductions in alkamide concentrations were found in dried, crushed *E. purpurea* stored at 20°C in the presence of light or at 30°C in the dark over 60 days, respectively (Wills and Stuart, 2000).

In addition to storage length, the method of drying plant tissue can have an impact on alkamide retention. Freeze-dried *E. purpurea* roots had significantly higher total alkamide levels compared to vacuum (50 mmHg) microwave-dried, air-dried at 70°C, and vacuum microwave-dried at partial vacuum (200 mmHg) products Kim *et al.* (2000a). In the partial vacuum-dried samples, the total alkamides and tetraenoic acid isobutylamides were significantly lower than other dried samples. In contrast, the total alkamide concentrations were higher in the air-dried (50°C) *E. purpurea* leaves than for the freeze-dried and vacuum microwave-dried samples.

Bauer (1999b) found that the alkamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, level was influenced by the preparation method. Nonthermal preparations appeared to have slightly higher levels of the tested alkamide than thermally treated products. Thus, the drying process may not be the best method for preparing *Echinacea* products. Pressing of the plant material to obtain an expressed juice is a common preparation method; however, preservation of the juice with ethanol is required. Direct ethanol extraction of the plant material can be used in place of the pressing operation.

Stuart and Wills (2000b) evaluated a series of ethanol:water mixtures as well as solvent:solute ratio and extraction temperatures as a means to identify the optimal solvent extraction conditions. These authors found that high ethanol levels favored alkamide extractions and that the 90:10 (ethanol:water v/v) removed 70 and 50% of the alkamides in the roots and aerial parts, respectively (Figure 5). In general, higher alkamide recovery was reported for the root tissue as compared to the aerial tissue. Although the alkamide recovery was quite high, the phenolic content of the extract was poor suggesting that only optimal extraction conditions were met for the alkamides. In general, the authors suggested a solvent ratio of 60:40 (ethanol:water v/v) for optimal extraction of alkamides and phenolic compounds. Dropping the ethanol content from 90 to 60% resulted in a 9% reduction in alkamide extraction (Stuart and Wills, 2000b). These observations were similar to the findings of Bergeron *et al.* (2000) and Binns *et al.* (2002b) who reported that a 70% ethanol solution was optimal for extracting various *Echinacea* phytochemicals; however, 95% ethanol was ideal for extracting alkamides. Sun *et al.* (2002) reported that the presence of

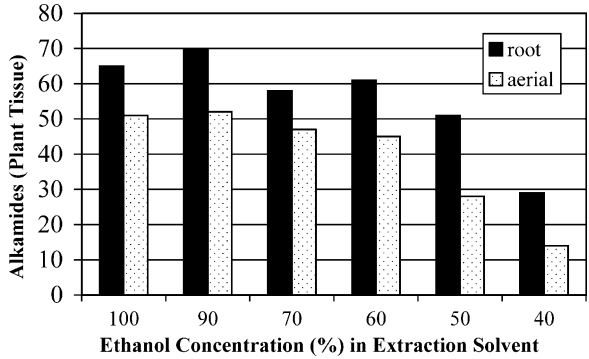

FIG. 5 The effect of ethanol concentration on alkamide recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

water hindered the extraction of alkamides by supercritical fluid (SF) carbon dioxide. However, no difference in alkamide extraction was noted for roots having moisture contents of 8.4 and 4.9%. They hypothesized that the presence of water (75%) in the fresh *Echinacea* sample altered the polarity of the supercritical carbon dioxide, thus reducing the extraction of the lipophilic alkamides.

The ratio of solvent:solute is also important as noted by Stuart and Wills (2000b). They noted that as the solvent:solute ratio increased from 2:1 to 8:1, alkamide in the extract increased from 37 to 68% for the roots and from 27 to 53% for the aerial parts. As the ratio of solvent:solute increases, so does the ethanol level thus enhancing alkamide recovery. In SF extractions, the addition of ethanol (10–12%) as a modifier to the supercritical carbon dioxide enhances the total extract or oleoresin yield but does not significantly influence the alkamide concentrations (Catchpole *et al.*, 2002). Sun *et al.* (2002) also noted that a 5% ethanol addition did not significantly affect the alkamide recovery.

Stuart and Wills (2000b) also reported that an extraction temperature of 20°C was optimal for extracting alkamides when a 60:40 ratio of ethanol:water was used as the solvent. Under these conditions, 61 and 45% of the alkamides were extracted from the roots and aerial tissues, respectively (Figure 6). In contrast, Sun *et al.* (2002) reported the higher temperatures favored alkamide extraction during SF extraction of *E. angustifolia* under constant pressures. These authors estimated that 2–3 times more alkamides could be extracted as the temperature of the SF extractor increased from 45 to 60°C, regardless of the vessel pressure. Catchpole *et al.* (2002) also reported similar findings in that increasing the temperature favored alkamide extraction when constant pressures were maintained in the SF extractor.

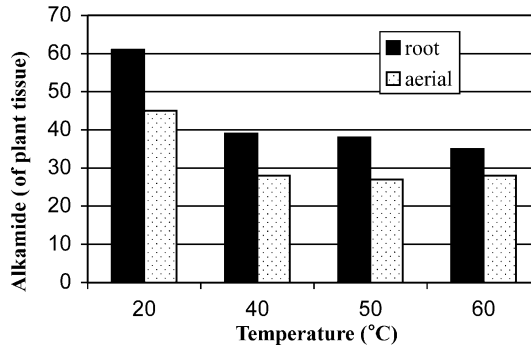

FIG. 6 The effect of temperature on alkamide recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

Stuart and Wills (2000b) reported a decrease, from 60 to 15%, in alkamide content of extracts prepared from *Echinacea* root with increasing particle size, from 300 to 4000 μm. Particle size is also important in SF extractions as noted by Sun *et al.* (2002). These authors noted a 10-fold increase in alkamide extraction simply due to grinding the sample.

C. CAFFEIC ACID PHENOLS

1. Concentrations and composition

Caffeic acid phenols (CAP; Figure 2) make up the largest percentage of the *Echinacea* phenolic compounds with flavonoids, phenolic acids, and anthocyanins contributing a smaller percentage. The tartaric acid derivatives of the CAP are believed to be responsible for some of the immune-enhancing activity of *Echinacea*, with cichoric (also referred to as chicoric) acid being the most important (Bauer *et al.*, 1989). Chlorogenic acid, verbascoside and echinacoside are present in significant quantities but have no immune-enhancing activity. However, these compounds do possess antioxidant activity and are hence potentially important phytochemicals that could act as synergists to enhance the biological activity of *Echinacea*.

A qualitative determination of the CAP in *E. pallida* showed that the roots contain high echinacoside levels; whereas, cichoric acid was the predominant CAP in the flowers and leaves (Cheminat *et al.*, 1988). This qualitative evaluation is a reasonable approximation of the CAP distribution in plant tissue; however, the level of the individual CAP is dictated by the *Echinacea* species evaluated. Pietta *et al.* (1998) presented a qualitative evaluation on the CAP in *E. purpurea*, *E. pallida* and *E. angustifolia* using micellar electrokinetic chromatography (MEKC). These authors noted that echinacoside was present in the roots and aerial parts of the *E. pallida* and *E. angustifolia,* but not *E. purpurea*. Cichoric acid is the predominant CAP in *E. purpurea* roots and aerial parts but is virtually absent in all parts of *E. angustifolia* and roots of *E. pallida*. The presence of other CAP in plant tissues is variable and species-variety-dependent (Tables III–V).

Becker and Hsieh (1985) reported that the roots and aerial parts contained 7.6 and 13 mg cichoric acid/g dried plant material, respectively. Bauer and colleagues reported that the range of cichoric acid levels in aerial parts was 1.2–3.1% and, in roots, 0.6–2.1% for German-grown *Echinacea* (Bauer *et al.*, 1988a; Bauer and Wagner, 1990). Wills and Stuart (1999) reported that the average cichoric acid levels for Australian *E. purpurea* roots and aerial parts were 13.2 (1.32%) and 12.9 mg/g (1.29%), respectively. Furthermore, chromatographic separation indicated that cichoric acid accounted for 63 and 67% of the phenols in the root and aerial parts, respectively. Similar to the

TABLE III
AVERAGE CICHORIC ACID CONTENT (mg/g PLANT PART DRY WEIGHT) OF VARIOUS PLANT PARTS OF *E. PURPUREA*

	Plant part			
Location	Roots	Flower	Leaf	Stem[a]
Stuart and Wills (2000a)	21	34	19	7.5
Becker and Hseih (1985)	7.5	13[b]	–[b]	n/r
Bauer and colleagues[c]	14	22	10	4
Binns *et al.* (2002a)[d]	8.3	6.6[e]	n/r	n/r

[a]Vegetative and reproductive stem; n/r = not reported.
[b]Reported as aerial parts but may include flower, leaf and stem parts.
[c]Bauer *et al.* (1988a) and Bauer and Wagner (1990).
[d]Binns *et al.* (2002a)—average cichoric acid content for *E. purpurea* grown under cultivated and wild environments.
[e]Reported as inflorescences (flower and reproductive stem parts).

alkamides, the *Echinacea* samples obtained from areas north of the 32°S latitude in Australia had higher levels of cichoric acid (Wills and Stuart, 1999). Fifty percent (50%) of the 62 samples tested had cichoric acid levels in the range 10–15 mg/g roots and 90% fell in the range 5–25 mg cichoric acid/g root material. In addition, 90% of the herbal samples had cichoric acid levels in the range 5–20 mg/g dried aerial parts (Wills and Stuart, 1999).

TABLE IV
CONTENT[a] AND DISTRIBUTION[b] OF CICHORIC ACID IN VARIOUS PLANT TISSUE OF MATURE *E. PURPUREA* GROWN OVER TWO SEASONS AT DIFFERENT LOCATIONS IN AUSTRALIA

	Location			
	Coastal		Tableland	
Plant Part	Year 1	Year 2	Year 1	Year 2
Roots	37[b]	31	35	44
Flower	24	32	17	7
Leaf	24	15	34	43
Stem	16	22	14	6
Total cichoric acid/plant[a]	1380	2120	847	2058

Adapted from Stuart and Wills (2000a).
[a]mg/plant dry weight.
[b]Contribution (%) to total cichoric acid/plant by each plant part.

TABLE V
AVERAGE TOTAL CAFFEIC ACID PHENOL CONTENT (mg/g DRY ROOT) OF VARIOUS *ECHINACEA* SPECIES AND VARIETIES GROWN UNDER CULTIVATED AND WILD ENVIRONMENTS[a]

	Germlings[b]	Wild-harvested	Wild-transplant
E. purpurea	10.5	8.0	6.5
E. pallida var *angustifolia*	12.9	3.2	2.0
E. pallida var *pallida*	2.5	2.5	0.5
E. pallida var *sanguinea*	0.2	1.0	0.5
E. pallida var *tennesseensis*	6.7	0.8	0.2
E. atrorubens var *atrorubens*	0.2	0.3	0.6

[a]Adapted from Binns *et al.* (2002a). Estimated total alkamide levels.
[b]Plants grown from wild-harvested seeds in a greenhouse.

Bauer *et al.* (1988a) noted that the *E. purpurea* had higher levels of cichoric acid than *E. angustifolia* DC. In addition, average cichoric acid contents in mg/g plant tissue of *E. purpurea* were 22, 14, 10 and 4 for the flower, root, leaf, and stem segments, respectively (Table III). The average cichoric acid content in Australian-grown *E. purpurea* was 34, 21, 19, and 8 mg/g dried flower, root, leaf, and stem segments, respectively (Stuart and Wills, 2000a). Binns *et al.* (2002b) also reported that the flowers had higher concentrations of cichoric acid than the roots of wild-harvested *Echinacea* obtained in the United States. Although the data suggests that geographic location may have a slight effect on cichoric acid concentration, the accumulation of cichoric acid in specific plant parts was not affected by location. In these studies, the flowers had the highest level of cichoric acid at all locations, followed by root, leaf, and stem segments (Table III).

E. purpurea grown in the coastal (32°S latitude, sea level, 13–23°C) and sub-tropic tableland (32°S latitude, 1030 m altitude, 13–23°C) growing sites in Australia had similar cichoric acid levels (Stuart and Wills, 2000a). The average cichoric acid levels were 34, 23, 20 and 9 mg/g plant part for the flowers, roots, leaves and stems, respectively, in mature *E. purpurea* plants grown in coastal areas. The tableland-grown, mature *E. purpurea* had average cichoric acid levels of 31, 18, 17 and 8 mg/g plant part for the flowers, roots, leaves and stems, respectively. In contrast, significantly higher levels of cichoric acid per plant were found in *E. purpurea* plants grown in coastal areas, due in part to the higher plant mass. Stuart and Wills (2000a) reported a total cichoric acid content of 1750 and 1452 mg/plant for *E. purpurea* grown in coastal and tableland areas, respectively (Table IV). The contribution of each plant part to the total cichoric acid level was similar between growing locations. The root tissue accounted for 37 and 35% of the total cichoric acid

in coastal and tableland grown plants, respectively, harvested at the mature stage of growth, followed by the stem (24 and 34%), flowers (23 and 17%), and leaves (16 and 14%). However, the contribution of each plant part harvested at the mature stage varied between two growing seasons (Stuart and Wills, 2000a). For example, the flower tissue accounted for 8% of the total cichoric acid levels in year one and 32% after the second growing season (Table IV). In general, cichoric acid levels decreased as the plant entered the senescent stage of the growing cycle (Stuart and Wills, 2000a). Kim *et al.* (2000b) noted a slight reduction in CAP in the samples harvested between August and October. Cichoric acid decreased significantly in freeze-dried samples between harvest periods, again indicating the importance of harvesting at the correct stage of the growing cycle. Perry *et al.* (2001) reported that cichoric acid contents were highest in summer and lowest in autumn-harvested plants, thus supporting the trends observed by other researchers. The mean cichoric acid levels of 2.0 and 2.3 mg/g root or aerial parts, respectively, were found in *E. purpurea* during the summer harvest, but decreased to 1.7 and 0.34 mg/g root or aerial parts, respectively, in the autumn-harvested *E. purpurea* (Perry *et al.*, 2001). These studies indicate the importance of harvest time on CAP retention.

Binns *et al.* (2002b) determined the CAP levels in cultivated and wild populations of various *Echinacea* species and varieties. Similar to the alkamides, no clear trend was observed across all species and varieties under all growing conditions (Table V). The CAP concentration was highest in the flower tissue in most samples. The flowers of the wild-harvested samples had the highest CAP levels, followed by the transplants and lastly the germlings. In contrast, the roots of the germlings had the highest CAP levels followed by wild-harvested samples and lastly the transplants (Binns *et al.*, 2002b). With a few exceptions, the CAP concentration was highest in *E. purpurea* samples and ranged from 6.5 to 10.5 mg/g of root material. Cichoric acid was the predominant CAP in *E. purpurea* roots; whereas, echinacoside predominated in all the other species and varieties tested (Table V). Cynarin also accounted for a significant proportion of the CAP in root tissue of cultivated *E. angustifolia* and *E. pallida* var. *tennesseensis* but contributed to a lesser proportion of the CAP in the flowers of these varieties (Binns *et al.*, 2002b). Cichoric acid was the predominant flower CAP in all *Echinacea* samples evaluated except *E. atrorubens* var. *atrorubens*, where chlorogenic acid was the predominant CAP. Dietz and Bauer (2001) reported similar findings for this species but did not observe cichoric acid or echinacoside, as did Binns *et al.* (2002b). Other *E. atrorubens* varieties contained significant levels of chlorogenic acid, which supports the work of Bauer and Foster (1991) who noted the presence of chlorogenic acid and echinacoside, but no cichoric acid, in the roots of *E. atrorubens* var. *paradox*. Chlorogenic acid was also in

significant levels in the flowers of *E. angustifolia* and *E. pallida* (Binns *et al.*, 2002b). *E. purpurea* had a chlorogenic acid content at 125 μg/g; whereas, 26 μg/g was found in *E. angustifolia* (Glowniak *et al.*, 1996).

Phenolic acids are structurally similar to the CAP. A phenolic acid content of 190 and 870 μg/g was reported for *E. angustifolia* and *E. purpurea*, respectively (Glowniak *et al.*, 1996). Caffeic acid is part of the structural features of the CAP but can also be found as a free acid. Binns *et al.* (2002b) reported caffeic acid levels of 10 and 70 μg/g root in *E. purpurea* and *E. pallida* var. *tennesseensis*, respectively. These authors also noted that *E. pallida* var. *tennesseensis* contained 70 μg caffeic acid/g flower. However, substantially higher (320 μg/g) caffeic acid levels were reported in the aerial parts of *E. purpurea* (Glowniak *et al.*, 1996). Vanillic acid was present in the aerial parts of *E. angustifolia* but not *E. purpurea*. In contrast, 86 μg of vanillic acid/g roots of *E. purpurea* was found using an analytical method which utilized MEKC (Pomponio *et al.*, 2002). Protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and ferulic acids were common to both *E. angustifolia* and *E. purpurea*.

2. *CAP isolation and key structural features*

The extraction of CAP can be completed using alcohol or alcohol/water mixtures (Bauer *et al.*, 1988b; Cheminat *et al.*, 1988; Wills and Stuart, 1999; Bergeron *et al.*, 2000). A common extraction approach uses a Soxhlet apparatus, methanol, and an extraction time of 12–24 h. Cheminat *et al.* (1988) completed an in-depth structural evaluation of 12 CAP isolated from *Echinacea* using NMR and grouped the CAP as quinyl esters of caffeic acid, tartaric acid derivatives, and phenylpropanoid glycosides. This grouping can be advantageous to individuals interested in obtaining standards because preliminary fractionation can be based on groups of similar compounds instead of single components. Recently, a chemical synthesis of cichoric acid has been reported (Lamidey *et al.*, 2002), thus providing an alternative source of cichoric acid for biological and stability studies.

The extraction and separation of the CAP can best be accomplished using the protocol of Cheminat *et al.* (1988), unless a preparative HPLC is available for separations. In this protocol, *Echinacea* was extracted with methanol: water (4:1) for 12 h followed by removal of methanol and extraction of the remaining aqueous phase with petroleum ether and chloroform. The aqueous phase was acidified, extracted with ethyl acetate and then *n*-butanol. After concentration of the ethyl acetate and *n*-butanol fractions, CAP were fractionated over a Sephadex LH20 column using methanol:water (4:1) followed by separation over silica gel, TLC or HPLC. Unlike other CAP,

cichoric acid can be isolated in a purified form after separation on Sephadex LH20 column. Echinacoside isolation can be achieved using a polyamide column and eluting with water followed by ascending methanol concentrations.

In our laboratory, we use a series of solvents to extract compounds with similar physical properties. Typically, an extraction protocol includes extracting the dried root or aerial parts with hexane to remove alkamides followed by 100% ethanol and 70% ethanol for the removal of phenolic and phenolic glycosides, respectively (Hall *et al.*, 2001). Bergeron *et al.* (2000) reported that 70% alcohol solutions were optimal for extracting the various *Echinacea* phytochemicals. A unique feature of their method was the use of ultrasound promoted extractions, which provided similar alkamide but higher CAP than traditional Soxhlet. Unlike Cheminat *et al.* (1988), we (Hall *et al.*, 2001) used reverse-phase or C_{18} packing material as the stationary phase for the separation of the CAP and a mobile phase of ethanol and 0.5% acetic acid under gradient elution. Bergeron *et al.* (2000) reported the use of reverse-phase stationary phase and gradient elution with methanol and water to purify echinacoside. Johnson *et al.* (2002) reported a three-phase method for concentrating an ethanol:water extract of *Echinacea*. In phase one, ethanol was removed via pervaporation followed by precipitation of the alkamides by microfiltration and osmotic distillation.

The variation in NMR data between CAP is due to the non-caffeic acid moiety and should then be used as a part of the identification process. In general, the caffeic acid moiety will have chemical shifts between 6.22 and 7.65 ppm with the aromatic proton ranging from 6.81 to 7.08 ppm. The hydrogens on the carbons neighboring the aromatic ring have chemical shifts near 7.59 ppm. The olefin hydrogens on the carbons next to the carbonyl have chemical shifts around 6.25 ppm, and are readily observed by the large associated coupling constants ($\sim$16 Hz) (Becker *et al.*, 1982; Becker and Hsieh, 1985; Cheminat *et al.*, 1988). The tartaric acid moiety is characterized by a proton resonance at 5.67 ppm. The elimination of one caffeic acid molecule from the tartaric acid moiety (i.e., caftaric acid) results in two chemical shifts at 4.57 and 5.34 ppm.

Echinacoside contains a caffeic acid, a β-(3,4-dihydroxyphenyl)-ethoxy substitution, two glucose groups, and a rhamnose group. The caffeic acid moiety has chemical shifts similar to those listed above while the aromatic proton chemical shifts for the β-(3,4-dihydroxyphenyl)-ethoxy substitution are found at 6.54, 6.66, and 6.67 ppm. The ethyl hydrogens on the carbon attached to the aromatic ring produce a signal at 2.73 ppm, whereas chemical shifts of 3.67 and 3.92 ppm are characteristic of protons on the carbon attached to the glucose moiety. For the glucose and rhamnose moieties, the majority of chemical shifts are between 3.14 and 3.75 ppm.

Fast atom bombardment mass spectrometry (FAB-MS), in the negative-ion mode, was used to elucidate the structure of CAP (Facino *et al.*, 1993). The negative ions ($[M - H]^-$) of 473, 353, and 311 *m/z* correspond to cichoric acid (MW 474), chlorogenic acid (MW 354) and caftaric acid (MW 312), respectively. FAB-MS mass spectrometry (FAB-MS/MS) of cichoric acid produced $[M - H]^-$ daughter ions of 311, 293, 179, 149 and 113 *m/z*. Cleavage of the ester bond results in the release of caftaric acid and a caffeoyl residue (160 *m/z*). The subsequent loss of a second caffeoyl or caffeic acid results in 149 and 113 *m/z* ions, which correspond to the tartaric acid moiety. Chlorogenic acid is a quinic acid ester of caffeic acid, thus, one would expect the loss of a caffeoyl unit. A daughter ion at 191 *m/z* represents the loss of caffeic acid from chlorogenic acid to give quinic acid. Minor ions at 309 and 147 *m/z* are indicative of a loss of carbon dioxide (44 *m/z* units) from chlorogenic acid and quinic acid, respectively. Other CAP can be evaluated using a similar approach.

3. Role of processing on CAP recovery and stability

A 1,2-dihydroxy moiety is a structural feature of molecules susceptible to the enzymatic degradation by polyphenol oxidase (PPO). Cichoric acid contains this structural feature and is thus susceptible to enzymatic degradation (Bauer, 1997). The immunostimulatory activity of *Echinacea* is partly due to the cichoric acid, and hence protection against enzymatic degradation is critical for retaining the potency of *Echinacea* preparations.

Cichoric acid content of six commercially available *E. purpurea* expressed juice preparations, preserved with ethanol (20%) or by thermal processing, varied significantly between preservation methods (Bauer, 1999b). The thermally treated preparations had an average cichoric acid content of 0.24 g/100 ml of extract. In contrast, the cichoric acid content of the five ethanol preserved preparations was 0.075 g/100 ml extract. Several preparations had no cichoric acid whereas one ethanol-preserved preparation contained 0.14 g cichoric acid/100 ml extract. Within this preparation, the cichoric acid content of several lots varied from 0 to 0.34 g/100 ml extract. Bauer (1999b) hypothesized that the inactivation of PPO by heat may account for the difference in cichoric acid content found between heated and non-heated preparations. Any expressed juice would be expected to contain enzyme inherent to the plant tissue; thus, additional processing of the expressed juice preparation is critical for CAP retention.

Kreis *et al.* (2000) characterized a PPO from the aerial part of *E. purpurea* as a diphenolase with a high affinity for caffeic acid. In addition, the PPO lacked monophenolase activity. The PPO was reported to be a 47–54 kDa protein having an optimal activity near pH 6.0 and reversibly inhibited by

metal-chelating agents. Copper was found to be essential for activity as the addition of excess copper could reverse the inactivation by metal chelators. Bergeron *et al.* (2002) noted that the addition of the metal chelators, citric and malic acids, greatly enhanced the stability of CAP in glycerin extracts of *E. purpurea*. In the control (i.e., without chelators) glycerin extracts, approximately 50, 72 and 80% reductions in caffeic, caftaric and cichoric acids were found, respectively after 4 months of storage; whereas a 21% increase in 2-*O*-feruloyl-tartaric acid was found during the same storage period. Citric and malic acids were found to be the most effective in preventing CAP degradation at the 0.5% level. A hibiscus extract at 15% was also found to be an effective antioxidant (Bergeron *et al.*, 2002). Cichoric acid levels in hibiscus, citric acid and malic acid protected glycerin extracts decreased by 44, 32 and 31%, respectively after 4 months of storage, which is significantly better than the 80% loss observed in the control sample. Other CAP were protected by the addition of the metal chelators/antioxidants. Additional studies showed that the addition of ascorbic acid (AA) (50 mM) or soaking of the plant material in a nitrogen-rich environment prevented cichoric acid degradation (Nüsslein *et al.*, 2000). These authors also found that the degradation of cichoric acid was promoted by protein extracts of *E. purpurea* during incubation at 40°C whereas heat processing inactivated the protein extract.

Perry *et al.* (2001) reported 50% reduction of cichoric acid and other CAP within minutes after water had been added to ground roots and aerial parts. They noted that the extraction solvent of 70% ethanol did not promote degradation and theorized that the PPO had been denatured. Nüsslein *et al.* (2000) reported that the ethanol concentration (22%) typically found in alcohol-preserved preparations, plus ethanol levels as high as 40%, were not able to prevent cichoric acid degradation. However, the addition of AA to the ethanol extracts did reduce the rate of loss but did not inhibit the degradation completely. Within 1 week, cichoric acid levels dropped by 20%, and by 4 weeks of storage 70% of the cichoric acid was gone from extracts containing 22% ethanol and 100 mM AA. In contrast, 80 and 90% of the cichoric acid in 30 and 40% ethanol-preserved extracts containing AA remained after a 4-week study, respectively. Nüsslein *et al.* (2000) recommended that 40% ethanol and 50 mM AA be used for the preservation of expressed juice products. They observed that the cichoric acid level in the chemically preserved preparations equalled those of thermal process preparations. In contrast, the addition of 30% ethanol and 50 mM AA to pressed juice was insufficient to inhibit the degradation of cichoric acid. Nüsslein *et al.* (2000) proposed that the degradation was caused by an esterase and not PPO. They observed an increase in caftaric and caffeic acids in stored extracts, indicating the hydrolysis of the ester bond between the caffeoyl

moiety and tartaric acid (Figure 7). If PPO were responsible for the degradation, one would not expect to find caffeic acid in the extracts because caffeic acid has been shown to be a very good substrate for PPO (Kreis *et al.*, 2000) and would undergo polymerization.

The loss of CAP during the growing season indicates that the enzymatic activity exists in the plant and that handling of the plant could further the degradation of CAP. Thus, handling of the plant is critical if high levels of CAP are to be retained. Post-harvest handling of *Echinacea* is as important for the production of dried products as is the thermal or extraction process is to pressed juice products. *E. purpurea* processed using various drying methods showed that freeze drying and vacuum microwave drying of flowers had significantly higher total cichoric and caftaric acid levels compared to air drying (Kim *et al.*, 2000b). Significantly higher CAP were retained in the samples air-dried at 40°C than at 25 and 70°C. Li and Wardle (2001) reported that the cichoric acid levels were significantly higher in *E. purpurea* dried at 45°C than at 40 or 35°C. Similar results were reported for *E. pallida* except that no significant differences in cichoric acid levels were found between 40 or 35°C. No correlation was found between the drying temperature and retention of echinacoside in dried *E. angustifolia* and *E. pallida* roots. Compared to a freeze-dried control, no significant reduction in echinacoside level was found in the roots of *E. angustifolia* dried at 23°C (Kabganian *et al.*, 2002). However, a 28 and 55% reduction in echinacoside was found at dry temperatures of 30 and 60°C, respectively.

Wills and Stuart (2000) found that cichoric concentration was not significantly altered when *Echinacea* was subjected to various physical treatments that included cutting, compression or crushing. These results appear to contradict other reports (Nüsslein *et al.*, 2000; Perry *et al.*, 2001) regarding the instability of the cichoric acid after harvesting. However, the method of damage may not have been sufficient to cause cell disruption normally caused by the pressing operation. Subsequently, there may not have been sufficient substrate–enzyme interactions to promote a high degree of

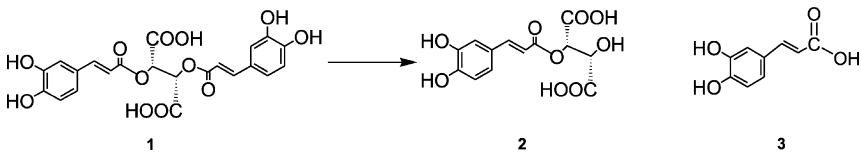

FIG. 7 The hydrolysis of the ester bond between the caffeoyl moiety and tartaric acid in cichoric acid (**1**) to give caftaric (**2**) and caffeic (**3**) acids as proposed by Nüsslein *et al.* (2000).

browning, although Wills and Stuart (2000) did observe some enzymatic browning. Kim *et al.* (2000b) reported that *Echinacea* product with a moisture content of 9.3% had a significantly lower cichoric acid content compared to the ones dried to 6.1% prior to storage. In contrast, Li and Wardle (2001) noted that cichoric acid levels were the greatest for *E. purpurea* samples having moisture contents of 10–15%. Although insignificant, higher echinacoside levels were reported for *E. angustifolia* roots dried to 10% moisture content. *E. pallida* roots dried to 5% moisture had significantly lower echinacoside levels than those dried to 10–15%.

Storage of freshly harvested *Echinacea* at 20°C and 60% RH did not significantly affect the concentration of cichoric acid over a 30-day storage period (Wills and Stuart, 2000). However, an 80% reduction in cichoric acid was reported for samples stored at 5°C and 80% RH. Although the storage temperature was significantly lower, the higher RH was thought to be the main reason for the enhanced cichoric acid degradation. For example, the moisture content of dried *Echinacea* stored at 20°C and 60% remained constant (10%) over the course of 60 days. In contrast, the moisture content of the dried sample stored at 5°C and 80% RH increased from 10 to 25% over the 60-day storage (Wills and Stuart, 2000). These authors proposed that drying *Echinacea* to a moisture content of 10–12% was critical for inhibiting enzyme activity. Alternatively, blanching prior to storage at 5°C and 80% RH can be an effective method for preserving cichoric acid (Wills and Stuart, 2000). Storage after 7 months showed that powdered extracts of *E. purpurea* roots were more stable than a 55% ethanol preparation (Livesey *et al.*, 1999). In powdered samples, cichoric acid was not significantly affected during 7 months of storage. However, samples stored at higher temperatures did contain lower levels of cichoric acid. For example, the cichoric acid level was 27% lower in samples stored at 25°C as opposed to storage at −20°C. In contrast, storage of the extracts was significantly affected by storage temperature. The greatest cichoric acid reduction (78%) occurred in the samples stored at 40°C (Livesey *et al.*, 1999).

Stuart and Wills (2000b) and Bergeron *et al.* (2000) evaluated several extraction techniques and conditions that involved a series of alcohol:water mixtures. Both groups of researchers found that alcohol levels of 60–70% favored CAP removal. Cichoric acid was extracted slightly more efficiently by 70% methanol than 70% ethanol but both solvents were significantly more efficient extraction solvents than their corresponding 100% counterparts (Bergeron *et al.*, 2000). Ultrasonic-promoted extractions significantly enhance cichoric acid extraction when 70% alcohol was used as the solvent (Table VI). In contrast, a reduction in cichoric acid extraction was found in the ultrasonic-promoted extraction using 100% alcohol solvents. Echinacoside recovery was not significantly affected by solvent type or extraction condition (Table VI).

TABLE VI
COMPARISON BETWEEN SOXHLET AND ULTRASOUND-PROMOTED EXTRACTION OF CICHORIC ACID AND ECHINACOSIDE FROM *E. PURPUREA* AND *E. ANGUSTIFOLIA*, RESPECTIVELY

Extraction condition	*E. angustifolia* roots Echinacoside	*E. purpurea* root Cichoric acid	*E. purpurea* top Cichoric acid
Soxhlet[a]—100% MeOH	14.0[b]	19.5	19.0
Ultrasound[c]—100% MeOH	13.5	12.0	14.0
Ultrasound—70% MeOH	14.0	43.0	30.0
Ultrasound—70% EtOH	13.5	38.0	27.0

Adapted from Bergeron *et al.* (2000).
[a]Soxhlet—1 h extraction in methanol (MeOH).
[b]mg/g tissue (approximations).
[c]Ultrasound—sample extracted three times using methanol or ethanol (EtOH) under 5 min exposure to ultrasound.

Stuart and Wills (2000b) reported that 60% ethanol was optimal for recovering cichoric acid from the roots and aerial parts of *E. purpurea*. These authors reported that cichoric acid accounted for 37% of the material in the 60% ethanol extract for both the root and aerial parts, but only 23–25% of the extract obtained from 70% ethanol extraction. Significant degradation of cichoric acid was noted as the extraction residues contained very little cichoric acid. The only exception to this trend was in the residues of the samples extracted with 100% ethanol (Stuart and Wills, 2000b). The denaturation of PPO probably contributed to the stability of cichoric acid in the residue. Enzyme inactivation further supported the observed increase in cichoric acid recovery as temperatures during the extraction increased from 20 to 60°C (Figure 8). Furthermore, cichoric acid recoveries were enhanced by increasing the solvent:solute ratio from 2:1 to 8:1 and by reducing the particle size from 4000 to 300 μm (Stuart and Wills, 2000b).

In contrast to alkamides, alternative extraction solvents such as SF carbon dioxide appear to be ineffective as an extraction solvent for CAP removal (Catchpole *et al.*, 2002; Sun *et al.*, 2002). Conditions evaluated by these researchers include pressures of 31–55 MPa and temperatures between 41 and 60°C. In both studies, ethanol was used as a solvent modifier, but the supercritical carbon dioxide was not modified sufficiently to promote the extraction of CAP. The addition of 10% methanol to the supercritical carbon dioxide at 25 MPa and 60°C was sufficient to promote the extraction of rosmarinic acid, a compound with similar structure features as cichoric acid (Bicchi *et al.*, 2000). Thus, additional work is needed to determine if SFE can be used as a method to remove CAP.

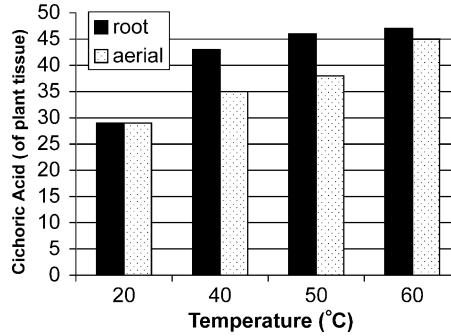

FIG. 8 The effect of temperature on cichoric acid recovery from *Echinacea* tissue (adapted from Stuart and Wills (2000b)).

D. GLYCOPROTEINS AND POLYSACCHARIDES

1. Concentrations and composition

Unlike the alkamides and CAP, the knowledge regarding concentrations and composition of glycoproteins and polysaccharides is limited. The roots of *E. purpurea* and *E. angustifolia* contain several glycoproteins in the 10–70 kDa MW range and recently a 1200 kDa glycoprotein has been isolated (Bauer, 1999a, 2000; Classen *et al.*, 2000). The glycoproteins of molecular weights, 17, 21 and 30 kDa comprised approximately 3% protein in which aspartate, glycine, glutamine and alanine are the predominant amino acids (Beuscher *et al.*, 1987). The sugar moieties include arabinose (64–84%), galactose (1.9–5.3%) and glucosamine (6%) (Bauer, 1999a, 2000). Classen *et al.* (2000) determined that the 1200 kDa glycoprotein was an arabinogalactan-protein (AGP) with a protein content of approximately 7%. Serine (16%) was the most abundant amino acid followed by alanine, hydroxyproline, and aspartate (Table VII). These authors also estimated that the AGP made up about 0.4% of the dry weight of the pressed juice. The AGP was approximately 83% polysaccharide with galactose and arabinose accounting for 90% of the monosaccharides (Table VIII). Reconstruction of the purified AGP indicated that the carbohydrate moiety had an arabino-3,6-galactan structure. The backbone of the polysaccharide consisted of β-D-galactopyranosyl residues with a high degree of branching at the C-6 oxygen via (1 → 6) to β-D-galactopyranosyl containing terminal α-L-arabinofuranosyl residues (Classen *et al.*, 2000). These authors estimated that 700 amino acid and 7000 glycosyl residues make up the structural features of the AGP

TABLE VII
AMINO ACID DISTRIBUTION[a] IN AN ARABINOGALACTAN-PROTEIN FROM *E. PURPUREA*

Amino acid	%
Serine	16.0
Alanine	12.5
Hydroxyproline	11.5
Asparagine/aspartic acid	10.6
Threonine	10.5
Glutamine/glutamic acid	9.4
Arginine	7.5
Glycine	4.6
Valine	4.1
Histidine	3.7
Lysine	3.3
Leucine	2.5
Isoleucine	2.2
Phenylalanine	1.6

Adapted from Classen *et al.* (2000).
[a]% of isolated protein.

isolated and that the galactose and hydroxyproline residues link the polysaccharide and protein moiety together.

The polysaccharides can be grouped into one of the three general categories that include fructans, pectic polysaccharides or arabinogalactans. A 35 kDa polysaccharide, 4-*O*-methyl-glucuronoarabinoxylan, isolated from the hemicellulosic fraction of *E. purpurea* was characterized by Wagner *et al.* (1984, 1985) and Proksch and Wagner (1987). These authors noted that xylose, galactose and arabinose made up the largest proportion of the

TABLE VIII
MONOSACCHARIDE DISTRIBUTION[a] IN AN ARABINOGALACTAN-PROTEIN FROM *E. PURPUREA*

Monosaccharide	%
Galactose	59.1
Arabinose	33.2
Glucosamine	4.0
Mannose	2.6
Rhamnose	1.1

Adapted from Classen *et al.* (2000).
[a]% of isolated polysaccharide.

monosaccharides whereas glucose, mannose and rhamnose were minor constituents. An arabinose:xylose ratio of 1:5 and a 4-*O*-methyl-glucuronic acid:xylose of 1:5.7 were observed. β-(1 → 4)Xylopyranosyl residues make up the backbone of the acidic arabinoxylan, with branch points at C-2 and C-3 oxygens. Arabinofuranosyl residues are linked at the C-3 oxygen via α-(1 → 5) bonds while a β-linked 4-*O*-methyl-glucuronic occupied the C-2 location (Proksch and Wagner, 1987). Emmendörffer *et al.* (1999) stated that a second immunostimulating polysaccharide, a 50 kDa acidic arabinorhamnogalactan, was characterized from the herbal parts of *E. purpurea*. In this polysaccharide, the molar ratios of monosaccharides were 1:0.8:0.6:0.6 for arabinose, rhamnose, galactose and glucose, respectively.

An acidic arabinogalactan (75 kDa) and two fucogalactoxyloglucans (10 and 25 kDa) have been isolated from the cell cultures of *E. purpurea* and were found to be characteristically different from the arabinogalactan from the *Echinacea* plants (Wagner *et al.*, 1988). An arabinose:xylose ratio of 1:1 and approximately 16% galacturonic acid were observed in the cell culture-derived arabinogalactan. Galactose accounted for 75% of the hexose sugars and was predominantly linked 1 → 3 to other galactose units to make up the polygalactan backbone. The β-D-galactan backbone was linked 1 → 6 to galactose at branch points on every second galactose. Approximately, 7 → 8 galactose residues in the side-chains are terminally linked 1 → 3 to arabinose. Other branch points may include a 1 → 2 or 1 → 4-linked rhamnosyl or galacturonic acid residue, respectively (Wagner *et al.*, 1988). These authors also noted that larger arabinan side-chains existed and that 1 → 5 linkages predominated. The fucogalactoxyloglucans both had the same molar ratios of glucose, xylose, galactose and fucose (1.5:1.0:0.4:0.1) and a 1 → 4-linked glucan backbone. Branch points were predominant (65%) at the C-6 oxygen. Recently, a hexasaccharide identified by Wagner *et al.* (1988) has been chemically synthesized (Csává *et al.*, 2001). For additional discussion on the polysaccharides, see Bauer (1999a, 2000) and Emmendörffer *et al.* (1999).

Inulin was identified as a major (5.9%) component of the roots of *E. angustifolia* (Heyl and Stanley, 1914). Giger *et al.* (1989) noted that polymerization of fructans occurred over the course of winter as observed by the reduction in fructose from October and May. The rate of polymerization was faster in *E. purpurea* than *E. angustifolia*, suggesting that other polysaccharides may develop in a similar manner. Additional research is needed to identify the effects of harvest time on polysaccharide composition.

2. *Glycoprotein and polysaccharide isolation and key structural features*

Of the phytochemical components in *Echinacea*, the glycoproteins and polysaccharide have been the least characterized. A general protocol

(Proksch and Wagner, 1987; Wagner *et al.*, 1988; Classen *et al.*, 2000) for separating the polysaccharides included pressing of the plant tissue, heating or acid hydrolysis, methanolysis, reduction and oxidation reactions. For complete NMR and sugar distribution data, see Proksch and Wagner (1987), Wagner *et al.* (1988), Classen *et al.* (2000) and Csává *et al.* (2001).

3. *Role of processing on polysaccharide recovery and stability*

In general, polysaccharides tend to be more stable than alkamides and CAP, but their presence in *Echinacea* preparations is somewhat dictated by processing conditions. For example, one would expect dried plant material to contain the highest polysaccharide levels, followed by expressed juice preparation and alcohol tincture. Limited data are available regarding the effects of post-harvest handling and processing on polysaccharide levels.

In the roots of *E. angustifolia* or *E. pallida*, the level of polysaccharides decreased as the drying temperature increased (Li and Wardle, 2001). Although the decrease in polysaccharide levels was not significant, a 7 and 15% reduction in polysaccharides was observed in *E. angustifolia* roots as the drying temperature increased from 35 to 40 or 45°C, respectively. In *E. pallida*, only 2 and 3% of the polysaccharides were lost as the temperatures increased from 30 to 40 or 45°C, respectively. In contrast, higher drying temperatures did not affect the polysaccharide retention in *E. purpurea*. There was a significant increase in polysaccharide levels as drying temperature increased from 35 to 40 or 45°C. For example, the concentration of polysaccharides increased from 24 to 28.6 mg/g root as the drying temperature increased from 35 to 45°C, (Li and Wardle, 2001). A reduction in polysaccharide content (dry weight basis) was noted in roots that had been dried to 15% moisture compared to 5%, regardless of the *Echinacea* species. For example, 1.3 times more polysaccharides were present in the roots of *E. pallida* dried to 5% moisture as opposed to roots dried to 15% moisture (Li and Wardle, 2001).

The low concentration of polysaccharides in alcoholic tinctures would be expected due to the insolubility of the polysaccharides in ethanol. Solvents containing approximately 50% v/v ethanol can be used to precipitate polysaccharides (Bauer, 1999a). Although no data were given, Bergeron *et al.* (2000) reported that 70% ethanol extracted lower polysaccharide levels than 70% methanol. A 65% glycerin extract had higher polysaccharides than a 50% ethanol extract (Bergeron *et al.*, 2002). In contrast to high alcohol levels, low to intermediate alcohol levels can be a viable extraction solvent for polysaccharides. Gahler *et al.* (2001) patented a method for extracting polysaccharides using 20% ethanol and a solvent temperature of 55°C. Ethanol concentrations greater than 20% and temperatures lower than 55°C reduced

polysaccharide recovery. An extract containing 120 mg polysaccharides/ml was produced using vacuum evaporation at 50 mbar and 60°C for 15 h (Gahler *et al.*, 2001).

III. STANDARDIZATION, QUALITY ASSURANCE AND REGULATIONS

A. IS THERE A NEED FOR STANDARDIZATION?

As already indicated throughout the chapter, the type and quantity of *Echinacea* phytochemicals vary significantly. A number of factors such as growing season, part of the plant utilized in the preparation of the commercial product, the species/variety (i.e., *purpurea*, *angustifolia* DC, *pallida*, etc.) of *Echinacea* used, method of harvest and processing can affect phytochemical concentrations in *Echinacea* products (Bauer, 1997). Bauer (1996) stated that the products should be classified according to plant species and processing methods. Bauer (1999b) noted differences in cichoric acid and alkamide concentrations between thermally treated and ethanol-preserved preparation. A survey of 46 samples from 25 commercial brands of *Echinacea* showed that alkamides varied significantly among products (Osowski *et al.*, 2000). These authors found that the alkamides and cichoric acid ranged from 0 to 60.5 μg/ml and from 0 to 4600 μg/ml, respectively. Tablets and pressed juice products had the greatest variations in cichoric acid whereas homeopathic tinctures had the greatest alkamide variation. Similar observations, with regard to product variability, were found in an Australian study that evaluated 32 *Echinacea* products (Wills and Stuart, 1998). For dry products (i.e., tablets, capsules), the cichoric acid content ranged from 0.2 to 6 mg/ml (200–6000 μg/ml) whereas liquid products obtained from retail outlets and naturopaths contained 0–3.9 and 0.1–4.7 mg/ml, respectively. The range in alkamide concentrations was similar among products; however, the average alkamide content was 1.7 times higher in samples obtained from naturopaths than from retail outlets (Wills and Stuart, 1998). These authors also noted that 28% of the samples had alkamide levels near zero; whereas, 16% of the samples had cichoric acid levels near zero, suggesting that alkamides may be more prone to degradation.

The variation in phytochemical levels between *Echinacea* preparations is apparent. Many researchers have suggested that standardization of phytochemicals in *Echinacea* products is needed. However, standardization of the products is difficult due to the complexity of the phytochemicals, the lack of understanding regarding interaction between individual phytochemicals

and the lack of agreement as to which phytochemical(s) should be used for standardization.

B. QUALITY ASSURANCE OF *ECHINACEA* PRODUCTS

1. Standardization

The CAP are commonly used as marker compounds and labeled as "phenolics" on many dietary supplements. However, general spectroscopic methods used for determining total phenols can sometimes give erroneous readings. Targeting specific compounds would be more advantageous and provide additional information such as plant species/variety and parts of the plants used in the preparation.

Echinacoside has been used to standardize many preparations, but the lack of immune-enhancing activity of this component suggests that it should not be used as a component for standardization. However, this compound could be used as a marker compound for authenticating the species of *Echinacea* used in the preparation. For example, the lack of echinacoside in an extract would indicate that *E. purpurea* would be the species most likely used in that preparation. The standardization of *Echinacea* products based on cichoric acid and alkamides has been proposed, due to the biological activity of these compounds (Bauer, 1999a,b; Perry *et al.*, 2000). Binns *et al.* (2002c) used the statistical method, canonical discriminant analysis, to differentiate growing locations among nine populations of *E. angustifolia*. One population was characterized by having traces of cynarin, and three alkamides and high amounts of echinacoside and tetradeca-8*Z*-ene-11,13-diyn-2-one (a marker ketone for *E. pallida*). Binns *et al.* (2002c) speculated that this population may have been a hybrid between *E. pallida* (Nutt.) and *E. angustifolia* DC. This statistical tool was able to distinguish between growing locations and was able to differentiate species/varieties. Additional work should be done using this statistical method to determine differences among other population of *Echinacea*, as a method to differentiate commercial products, and eventually to determine the relationships between potency and active constituents. Until additional research can pinpoint the specific phytochemical(s) and biologically active concentrations, no uniform standardization method will be used by all manufacturers. However, there is a growing consensus among researchers that CAP (specifically cichoric acid) and alkamides should be targeted as the phytochemicals for standardization. Polysaccharides do have biological activity; however, the analytical methods have not been sufficiently optimized for routine quality assurance evaluations. In addition, polysaccharides are often absent in alcohol-based products.

2. *Analytical methods for quality assurance*

A reverse-phase HPLC method can be routinely used for evaluating CAP and alkamides in *Echinacea* preparations. If dried products are evaluated, the CAP and alkamides must first be extracted prior to HPLC analysis. In contrast, some liquid preparations may need to be concentrated for example, via solid-phase extraction, prior to analysis. We have found in our laboratory that a sequential extraction of dried products with hexane (100%), ethanol (100%), and lastly ethanol:water (70:30 v/v) was an effective method to fractionate the various phytochemicals (Hall *et al.*, 2001). An alternative extraction protocol using only 70% ethanol as the extraction solvent has been reported (Bergeron *et al.*, 2000). In some cases, concentration of the alkamides may be needed prior to HPLC analysis. A separation of alkamides from CAP can be completed using C-18 solid-phase extraction columns (Schieffer, 2000; Schieffer and Kohn, 2002). Briefly, the CAP elute from the column in the methanol:water (30:70 v/v) solvent while the alkamides are removed using acetonitrile:water (90:10 v/v). Liquid preparations with low alkamide concentrations are ideally suited for this method because this technique concentrates the alkamides, thus increasing the sensitivity. However, the simultaneous analysis of CAP and alkamides using HPLC-photodiode array-electrospray mass spectrometry (Luo *et al.*, 2003) may hold promise as a rapid analytical method for analyzing the phytochemical constituents.

The method of Bauer (1999b) illustrated the most common method to evaluate alkamides using reverse-phase HPLC. The separation was completed using a gradient elution of water (eluent A) and acetonitrile (eluent B) linearly from 40 to 80% eluent B at 1 ml/min over a C-18 reverse-phase column and detection at 254 nm. TLC using silica 60 plates with indicator ($F_{254\ nm}$) can be used as an alternative to HPLC. However, this method is less adaptable for large number of samples requiring a rapid turn-around typically associated with quality assurance programs. In contrast, a high-performance TLC (HPTLC) method is being evaluated as a rapid screening method for the identification of *Echinacea* species present in commercial products and potential adulterants (Reich *et al.*, 2002).

A reversed-phase (LiChroCART 125-4 column) system with gradient elution is an effective method for separating the CAP within 12 min (Bauer, 1999b). The gradient system included water plus 0.1% orthophosphoric acid (85%) as eluent A and acetonitrile plus 0.1% orthophosphoric acid (85%) as eluent B. A linear gradient from 10 to 30% eluent B was completed within 20 min at 1 ml/min and detection at 330 nm. Bergeron *et al.* (2000) modified the method to include a pH adjustment to 2.80 for a solvent system containing phosphoric acid (solvent A) and 1% phosphoric acid (1 M) in acetonitrile (solvent B).

Alternative methods for assessing *Echinacea* phytochemicals include the use of MEKC or near infrared reflectance (NIR) spectroscopy. Pietta *et al.* (1998) used a 25 mM tetraborate buffer containing 30 mM SDS at pH 8.6 for the separation of CAP. The MEKC separation was completed within 20 min with good resolution. Goti *et al.* (2002) reported a simultaneous separation of both the CAP and alkamides using MEKC technique and variable wavelength detection. The separation was completed in 10 min using a mobile phase of SDS (110 mM) and hydroxypropyl-β-cyclodextrin (100 mM) in Britton–Robinson buffer (10 mM, pH 8.0). The advantage of this method was that the cost of operation is less than HPLC, since organic solvents were not needed. Recently, several NIR spectroscopy techniques have been reported for the analysis of adulteration and cichoric acid (Laasonen *et al.*, 2002; Gray *et al.*, 2001). Laasonen *et al.* (2002) reported that NIR could discriminate between *E. purpurea* and 10% adulterated *E. purpurea* samples. These authors also noted that about 10% of the *E. purpurea* samples and 0% of the adulterated samples were misidentified. Gray *et al.* (2001) evaluated 169 root samples using NIR and found between 1.8 and 19.1 mg cichoric acid/g root. These authors cautioned that the partial least squares regression used in the calculations requires additional samples from various locations before the method can be adopted for routine cichoric acid determinations. Schulz *et al.* (2002) also reported the use of NIR to measure the echinacoside content in *E. angustifolia* and *E. pallida* roots.

Gas chromatography (GC)–MS coupled with multivariate statistical analysis proved valuable in verifying the authenticity of *Echinacea* species (Lienert *et al.*, 1998). Similar root extracts could be grouped, based on the identified compounds from the GC-run, by principal component and cluster analysis. The correct grouping of the *Echinacea* species (i.e., *purpurea*, *angustifolia*, and *pallida*) was not influenced by the extraction method or by the aging process of the roots.

C. PRODUCT REGULATIONS

Echinacea is sold as a dietary supplement in the United States and as a natural health product in Canada. However, Health Canada does support the use of *Echinacea* in food products; thus functional foods could be developed for the Canadian market. In the United States and Canada, there are no restrictions on the species/varieties used in products. In Germany and many European countries, *Echinacea* products are sold as drugs in pharmacies (Bauer, 2000). In addition, not all products are approved for use in all countries. For example, *E. purpurea* aerial parts and *E. pallida* roots are approved in Germany; whereas *E. angustifolia* and *E. purpurea* roots are not (Blumenthal, 1998).

The regulation of *Echinacea* products in the United States would fall under the "Dietary Supplement Health and Education Act of 1994" (DSHEA). In the United States, statutory 403 (a)(1) of the Federal Food, Drug, and Cosmetic Act "prohibits labeling that is false or misleading." Under DSHEA, a structure/-function claim can be made, provided the claim meets the criteria set forth in statutory 403(r)(6). Structure/function claims are permissible if a specific disease is not targeted, unless reviewed by FDA. A structure/function claim such as "supports the immune system" would be acceptable whereas "alleviates the common cold or flu" would not be acceptable for *Echinacea* because the claim targets a specific disease state (i.e., cold or flu).

Echinacea must first be approved as a GRAS ingredient before it can legally be added to food; thus functional foods containing *Echinacea* cannot be legally sold. Creating a functional food and marketing it, as a dietary supplement is also not legal if the food resembles traditional food such as soup. In many European countries, health claims are not permitted on food products while in other countries, such as, Japan, claims are allowed under the "Foods for Specified Health Use" (FOSHU) system. The benefits of *Echinacea* in laboratory studies show promise and potential functional foods be derived from this research provided the studies support the safety of *Echinacea*. Health Canada has explicit definitions for *Echinacea* products and labeling requirements (Health Canada, 1999).

One issue not resolved by the DSHEA, specifically for botanical or herbal dietary products, is the lack of standardization of the active component. As mentioned previously, the standardization of *Echinacea* products is based on the level of plant material rather than one specific compound. In many cases, the specific compound responsible for the health benefit has not been fully characterized. In addition, many phytochemical constituents may participate in an observed health benefit. The lack of standardized preparations may, in fact, be responsible for the conflicting reports surrounding the biological activity of *Echinacea*.

IV. BIOLOGICAL AND TOXICOLOGICAL ACTIVITIES

A. BIOLOGICAL ACTIVITIES

1. Immunological investigations

Echinacea is promoted as an immune-enhancing herbal product and could be easily incorporated into cereal-based products (Wilson, 1998) to create a functional food. There are over 300 research articles dealing with the biological activity of *Echinacea*. In addition, over two million prescriptions

are filled by German physicians annually (Barrett *et al.*, 1999), thus providing some evidence of the safety of *Echinacea* products. Before *Echinacea* can be considered as a component in functional foods, additional research is needed to support the biological activity in a clinical setting and to determine the adverse effects of consuming *Echinacea*. The following discussion highlights only a fraction of the studies completed to date. The author suggests the reviews of Bauer (1999a, 2000), Emmendörffer *et al.* (1999) and Melchart and Linde (1999) for more detailed information.

Improving immune response has been the most widely documented benefit of *Echinacea* (Vömel, 1985; Bauer *et al.*, 1988d; Erhard *et al.*, 1994; Burger *et al.*, 1997; See *et al.*, 1997; Rehman *et al.*, 1999). Most studies support the *in vitro* nonspecific immune-enhancing activity while *in vivo* studies are less convincing. The expressed juice of *E. purpurea* has been the primary plant preparation used in many of the biological investigations. Alcohol-diluted expressed juice and ethanol extracts of the plant are other preparations used in immune-enhancing studies. Specific pure compounds have also been used in some studies.

Natural killer (NK) cells are responsible for nonspecific immunity by targeting virus-containing cells or tumor cells via phagocytosis. Cytokines such as TNF-α and IL-1 enhance NK cell activity whereas prostaglandins are inhibitory (Currier and Miller, 2001). Thus, compounds that promote macrophages to secrete cytokines or compounds that inhibit prostaglandin production would be expected to enhance immunity. Phagocytosis (i.e., the first immune reaction against invading foreign substances) of erythrocytes was significantly enhanced upon treatment with extracts of *Echinacea* (Vömel, 1985). Coeugniet and Elek (1987) found that the pressed juice of *E. purpurea* enhanced nonspecific cell mediated immunity at concentrations between 1 and 100 μg/g, whereas 1000 μg/g treatments were ineffective. These authors noted that the 1000 μg/g concentration promoted leukocytes mortality but suggested that this concentration was clinically irrelevant. A dose-dependent increase in the percentage of phagocytic granulocytes was found after treatment of white blood cell suspensions with lyophilized expressed juice of *E. purpurea* (Stotzem *et al.*, 1992). Lyophilized juice at concentrations of 1 and 5 mg/ml was most effective whereas the phagocytic activity at the 12.5 mg/ml concentration was not significantly different from the control. Only the 5 mg/ml had a significantly higher phagocytosis (i.e., number of phagocytosed starch grains per granulocytes) index (Stotzem *et al.*, 1992). These authors did note that the lack of activity at the highest lyophilized expressed juice concentration (12.5 mg/ml) was due to a cytotoxic effect on the granulocytes. Gaisbauer *et al.* (1990) also supported the importance of concentration on phagocytosis. In contrast, no immune-enhancing activity was found in mice fed various *Echinacea* products (South and Exon, 2001).

Ethanol extracts of *E. purpurea* and *E. angustifolia* roots were also found to enhance immune indices (Erhard *et al.*, 1994; Currier and Miller, 2000). Erhard *et al.* (1994) determined the mean channel of fluorescence intensity, which is proportional to the number of latex particles ingested or adhering to single granulocytes as one measure of immune response. The second measure, phagocytosis index, was also determined as the difference between the average numbers of latex particles ingested by the test compound and those adhering to the granulocytes after treatment with cytochalsin D (a compound that inhibits ingestion of latex particles but not adhesion). Collectively, phagocytosis incorporated both measurements. Vitamin C and a root extract of *E. angustifolia* were the only two treatments that significantly enhanced phagocytosis (Erhard *et al.*, 1994). The addition of *Lachesis muta*, *Aconitum napellus*, or *Apis mellifica* extracts to *E. angustifolia* root extract further enhanced phagocytosis. Wagner and Jurcic (1991) also found enhanced phagocytosis when combinations of *Eupartorium perfoliatum*, *Baptisia tinctoria* and *Arnica montana* were added to *E. angustifolia*. In contrast, no immune-enhancing activity was noted in patients given an extract containing *E. angustifolia*, *Eupartorium perfoliatum* and *Thuja occidentalis* after curative surgery for different malignant tumors (Elsässer-Beile *et al.*, 1996).

Burger *et al.* (1997) reported that cytokine production was enhanced in peripheral blood macrophages after treatment with an ethanol extract from the aerial parts of *Echinacea*. A dose-dependent interleukin 1 (IL-1) production was not found within or among all the fresh pressed juice lots of *Echinacea* tested. However, the 1.20 μg/ml concentration in two lots did stimulate the macrophage production of IL-1 to a greater extent. A third lot of fresh pressed juice had a maximum IL-1 production at 0.20 μg/ml. In contrast, the highest concentration (10 μg/ml) of the dried juice produced the greatest levels of IL-1 (Burger *et al.*, 1997). In the fresh pressed juice products, the 0.05 μg/ml dose promoted the greatest TNF-α production whereas 10 μg/ml dose produced the greatest TNF-α level of a third lot. The 0.03 μg/ml concentration of dried juice gave the greatest TNF-α production. The composition of the extracts may account for the variability in cytokine production (Burger *et al.*, 1997). Additional work by these authors showed that, at low doses (0.012–0.1 μg/ml), a dose-dependent cytokine production by macrophages was apparent irrespective of the product tested (i.e., fresh pressed or dried juice).

Administration, via mouse chow, of *E. purpurea* root extracts at 0.45 mg/day per mouse was found to enhance the number of NK cells in healthy young and old mice (Sun *et al.*, 1999; Currier and Miller, 2000) and leukemic mice (Currier and Miller, 2001, 2002). The number of NK cells increased in both the spleen and bone marrow, and paralleled cell cytolytic

function (Sun *et al.*, 1999; Currier and Miller, 2000). In aged mice, the number of NK cells returned to levels typically found in younger mice (Currier and Miller, 2000). In the spleen, a 30% increase in NK cells was found in mice fed *Echinacea* root extracts for 14 days compared with the *Echinacea*-free diet (i.e., control). Currier and Miller (2001) found a 2.5-fold increase in the number of NK cells in the spleen after 9 days of *E. purpurea* administration. After 3 months, the number of NK cells was 2–3 times that of normal, nonleukemic mice. The addition of melatonin to the *Echinacea*-containing diet resulted in a decrease in the NK cell numbers in the spleen (Currier and Miller, 2001). In contrast, bone marrow NK cells returned to levels similar to nonleukemic mice when melatonin and *Echinacea* were co-administered in the diet. No leukemic mice versus treated mice remained alive by 3 months; thus, the administration of *Echinacea* significantly enhanced the survival of the leukemic mice. However, the addition of melatonin to the *Echinacea*-containing diet significantly increased the survival rate, with 50% of the leukemic mice surviving to and beyond 3 months. Further immune-enhancing activity was noted by a significant increase in NK cells when mice were immunized with dead leukemia cells 5 weeks prior to administration of *Echinacea* in the diet (Currier and Miller, 2002). See *et al.* (1997) reported that extracts of dried *E. purpurea* significantly enhanced the NK cells in healthy subjects and those suffering from chronic fatigue syndrome and acquired immunodeficiency syndrome, at concentrations greater than 0.1 μg/ml.

Combination of low doses of cyclophosphamide, *E. purpurea* extract (Echinacin®) and thymostimulin enhanced the immune functions of terminally ill cancer patients (Lersch *et al.*, 1990, 1992). NK cells increased between 17 and 29% whereas lymphokine activated killer cell activity increased from 180 to 195%. In contrast, polysaccharides isolated from cell cultures of *E. purpurea* did not enhance the phagocytic activity of granulocytes in chemotherapy patients (Melchart *et al.*, 2002).

Bauer *et al.* (1988d) found that ethanol extracts (1:10) of *E. purpurea*, *E. pallida* and *E. angustifolia* roots significantly enhanced *in vitro* phagocytosis of granulocytes. Partitioning of the ethanol extract of *E. purpurea* and *E. pallida* into chloroform and water gave lipophilic and polar fractions, respectively. The lipophilic fraction was significantly more active than the whole ethanol extract and the polar fraction at enhancing phagocytosis. Bauer *et al.* (1988d) hypothesized that the isobutylamides and polyacetylenes in the lipophilic fraction were responsible for the granulocyte phagocytosis. In addition, concentration of the extracts had varying degrees of activity. The ethanol extract of *E. purpurea* root was the most effective at 1 μg/g (10^{-4}% as reported by Bauer *et al.* (1988d)) whereas the 100 and 10 μg/g concentrations of the extract were most effective for *E. pallida*

and *E. angustifolia*, respectively. Irrespective of the *Echinacea* species/varieties, the 1 μg/g concentration of the lipophilic fraction obtained from the ethanol extract was found to have the highest activity. Furthermore, the *in vivo* granulocyte phagocytosis results correlated with the *in vitro* results (Bauer *et al.*, 1988d). Goel *et al.* (2002) reported that alkamides significantly enhanced (60%) the phagocytic activity of mice alveolar macrophages at a dose of 12 μg/kg/day. The phagocytic activity of the cells treated with 0.5 and 4 μg/kg/day levels were not significantly higher than the activity of the control. Significantly, more TNF-α and nitric oxide were generated by the alveolar macrophages treated with *Echinacea* components and stimulated, *in vitro*, with a lipopolysaccharide from *E. coli*. Again, the optimal concentration was 12 μg/kg/day for the alkamides (Goel *et al.*, 2002). However, the levels of TNF-α, interferon-γ (IFN-γ) and interleukin 2 (IL-2) released by rat splenocytes were not significant.

Polar constituents of *Echinacea* include polysaccharides and CAP. Wagner *et al.* (1985, 1988) reported that *in vivo* and *in vitro* experiments supported the immune-enhancing activity of a polysaccharide fraction. These authors reported that a polysaccharide fraction from *E. purpurea* herb and root enhanced phagocytosis of yeast particles by polymorphonuclear neutrophil cells (PMNC) by 27% at concentrations of 1 and 10 μg/ml, respectively. A 32% increase in phagocytosis by PMNC was reported after treatment with a 10 μg/ml polysaccharide solution obtained from *E. angustifolia*. Wagner *et al.* (1985) originally reported on *E. angustifolia*; however, Bauer (1999a) stated that *E. pallida* could possibly be the species/variety evaluated based on misidentification that commonly occurred prior to 1985. Carbon-clearance assay showed, in mice, that phagocytosis was enhanced at a dose of 10 mg/kg body weight, thus supporting *in vitro* observations (Wagner *et al.*, 1985). A 4-*O*-methyl-glucuronoarbinoxylan (35 kDa) was found to enhance phagocytic activity *in vitro* by 23% at concentrations as low as 10^{-4} mg/ml. However, no *in vivo* activity was observed (Proksch and Wagner, 1987). Polysaccharides isolated from cell cultures of *E. purpurea* were found to stimulate phagocytosis by macrophages, with the fucogalactoxyloglucan (25 kDa) having the greatest phagocytic activity (Wagner *et al.*, 1988). In addition, an arabinogalactan (75 kDa) stimulated TNF-α excretion from the macrophage (Wagner *et al.*, 1988). An arabinogalactan also stimulated the release of IL-1, TNF-α and IFN in macrophages, *in vitro* (Luettig *et al.*, 1989; Roesler *et al.*, 1991a). However, the same immune responses were not significantly enhanced after human subjects were injected intravenously with a polysaccharide solution, although nonspecific immune functions could be shown (Roesler *et al.*, 1991a). Goel *et al.* (2002) reported that a polysaccharide dosage

of 3000 μg/kg/day was required to induce a significant increase in TNF-α production by rat alveolar macrophage.

Another polar constituent, cichoric acid, did not enhance TNF-α production (Goel *et al.*, 2002) at levels up to 120 μg/kg/day. Schumacher and Friedberg (1991) noted that, over the concentrations (2.5–250 mg/kg) and delivery (injection, oral) methods tested, no immune-enhancing activity was found in mice given the lyophilized water extracts of *E. angustifolia*. In addition, the water-soluble CAP, echinacoside, was not found to enhance immune function over the concentrations of 1–250 mg/kg. Bauer *et al.* (1989) also reported that echinacoside, along with verbascoside and 2-caffeoyl tartaric acid, did not stimulate phagocytosis. However, these authors did find that cichoric acid was effective in stimulating phagocytosis at concentrations of 10^{-5} mg/ml.

To date, all the studies evaluating the immunostimulating activity of *Echinacea* have focused on extracts or purified compounds as they exist in nature. The conflicting results between *in vivo* and *in vitro* studies may be caused by the test system used in the studies. Rininger *et al.* (2000) developed an innovative approach to evaluate the activity of *Echinacea* preparation using an *in vitro* protocol designed to stimulate *in vivo* conditions. The unique feature of this protocol was that the *Echinacea* raw material was subjected to a simulated digestive process. The samples were first subjected to digestion by gastric juice for 2 h at 37°C, neutralized with sodium hydroxide and finally incubated in simulated intestinal fluid for 2 h at 37°C. Viability and proliferation of human peripheral mononuclear cells (PBMC) and release of cytokines from murine macrophages were used to characterize immunostimulatory activity. Rininger *et al.* (2000) reported a 10–15% variation in inter-assay TNF-α demonstrating a good reproducibility for the digestion assay. The production of TNF-α and nitric oxide by macrophages stimulated with digested *Echinacea* preparation were dose-dependent over the range 0–1280 μl/ml. Treatment of murine macrophages with various levels (5–320 μl/ml) of digested *Echinacea* preparation also promoted a dose-dependent secretion of other cytokines (IL-1, IL-6, IL-10). Various nondigested *Echinacea* products, including commercial products standardized for phenolics, at concentrations greater than 250 μl/ml were unable to stimulate cytokine production in murine macrophages (Rininger *et al.*, 2000). The viability and proliferation of PBMC was enhanced by digested *Echinacea* products only at concentrations found to stimulate cytokine secretion. This study suggests that the immune-enhancing components can survive the digestive process and possibly act as *in vivo* immunostimulating agents (Rininger *et al.*, 2000).

2. *Antimicrobial and antiviral activity*

Related to the immunostimulatory activity is the ability of macrophages and granulocytes to eliminate bacterial and viral infections. *Echinacea* has been found to promote resistance to viruses. Wacker and Hilbig (1978) found that mouse L 929 cells treated with methanol or aqueous extracts of *Echinacea* were 50–80 times more resistant to influenza, herpes and vesicular stomatitis viruses than nontreated cells. The treatment was effective for 24 h; however, the cells became sensitive to the viruses after 48 h suggesting that repeated treatment would be required. A dose-dependent antiviral activity of cichoric acid, echinacoside and caffeic acid was observed in mouse L 929 cells (Cheminat *et al.*, 1988). The authors also reported a substantial reduction in cell growth as the concentration of the components increased, with caffeic acid causing the most inhibition. The optimal cichoric acid concentration for reducing the infectiousness of vesicular stomatitis viruses by 50% was 125 μg/ml. Cichoric and caftaric acids inhibited the hyaluronidase enzyme; an enzyme produced by pathogenic organisms to penetrate tissue and cause infection (Facino *et al.*, 1995). Thus, this may partly explain the reduction in viral infections in the presence of *Echinacea*.

The CAP have been reported to inhibit HIV type 1 (HIV-1) integrase and HIV-1 replications at concentrations as low as 10 μM (McDougall *et al.*, 1998; King *et al.*, 1999; Reinke *et al.*, 2002). Cichoric acid inhibited 50% of the integrase activity and blocked HIV-1 infections by 50% at concentrations of 0.3 and 4 μM, respectively (Robinson *et al.*, 1996a,b). Hexane extracts of *Echinacea* roots were found to have antiviral activity against the Herpes simplex virus type 1 at a 0.12 mg/ml concentration (Binns *et al.*, 2002d).

Wildfeuer and Mayerhoer (1994) noted a 45% increase in phagocytosis of *Candida albicans* by granulocytes treated with *E. purpurea*. Macrophages eliminate *Listeria monocytogens* and *Candida albicans* from internal organs of animals after polysaccharides, isolated from cell cultures, of *Echinacea* were added to model test systems (Roesler *et al.*, 1991b; Steinmüller *et al.*, 1993). Roesler *et al.* (1991b) found that 1 mg/ml concentration of polysaccharides enhanced the production of reactive oxygen intermediates (ROI) by 270% in J774-macrophage cells. However, concentrations greater than 1 mg/ml suppressed ROI. Liver cell macrophages treated with 0.2 mg/ml of polysaccharide suppressed the growth of *Candida albicans* by approximately 95%. White blood cell counts were also found to significantly increase 2.5 h after injecting the mice with 0.2 mg of polysaccharides. The same polysaccharide dose was sufficient to protect 90 and 100% of the mice infected with a LD_{80} dose of *C. albicans* and *L. monocytogens*, respectively (Roesler *et al.*, 1991b). However, delaying the polysaccharide treatment for more than 18 h after administration of the microorganism to the mice was

ineffective in preventing animal death. A dose-dependent reduction in colony-forming units was reported in the spleen, liver and kidney after treatment with polysaccharides (Roesler *et al.*, 1991b; Steinmüller *et al.*, 1993).

The *in vitro* antifungal activity of the lipophilic extracts and commercial tinctures of *Echinacea* was enhanced after exposure to UV irradiation (Binns *et al.*, 2000). These authors noted that *E. purpurea* flowers and a commercial tea product were the only products that lacked antifungal activity after UV exposure. The photooxidation of the alkamide, ketoalkenes and ketoalkynes contributed to the enhanced antifungal activity (Binns *et al.*, 2000).

3. *Anti-oxidant and anti-inflammatory activities*

Rininger *et al.* (2000) noted that standardized *Echinacea* extracts to 3–4% phenolic acids did not necessarily equate to a greater radical scavenging activity. Product type appeared to have a greater impact on radical scavenging activity than the actual standardization process. Cichoric acid and other CAP were found to act as radical scavengers (Facino *et al.*, 1995). Although not tested, verbascoside and 2-caffeoyl-cichoric acid would be expected to have similar radical scavenging activity. Facino *et al.* (1995) reported that *Echinacea* protected collagen from free radical damage by scavenging the reactive oxygen species. Echinacoside and cichoric acid were found to prevent collagen degradation best followed by cynarin ≈ caffeicacid and chlorogenic acid. Alcohol extracts of roots had greater radical scavenging activity than the leaf extracts irrespective of *Echinacea* species/variety (Sloley *et al.*, 2001). However, *E. purpurea* had the highest radical scavenging activity among the three species. The radical scavenging activity of the alcohol extract was equivalent to 1–4% of that of pure AA. In contrast, pure cichoric acid has three times more scavenging activity than AA (Sloley *et al.*, 2001). Alcohol root extracts of *E. pallida* prevented malondialdehyde (MDA) formation in catecholaminergic neuroblastoma SH-SY5Y cells better than *E. purpurea* and *E. angustifolia*. However, the MDA levels were not significantly lower than that observed in the control. The addition of iron to the test system resulted in a significant increase in MDA formation for all extracts and the control. The increase in MDA in the *Echinacea*-treated cells was significantly lower than that of the control. Again, cells treated with *E. pallida* had the lowest MDA formation. Irrespective of the *Echinacea* species/variety, the concentration of 0.5 mg/ml prevented MDA formation more than the 1 mg/ml concentration in the iron-free test system whereas the opposite was true for the iron-containing test system (Sloley *et al.*, 2001). In contrast to the radical scavenging assay, the leaf extracts inhibited the formation of MDA greater than the root extracts. No clear trends were apparent with regard to *Echinacea* species/variety or concentration of the

additive having the greatest activity (Sloley *et al.*, 2001). However, Hu and Kitts (2000) reported that *E. angustifolia* and *E. pallida* were more effective than *E. purpurea* in various *in vitro* assays and suggested that the antioxidant capacity was due to the high levels of echinacoside.

The anti-inflammatory property of *Echinacea* has been associated with the alkamides and polysaccharides. However, a standardized extract containing 4% phenolic acids was found to be more effective at inhibiting prostaglandin synthesis than the *E. purpurea* herb (Rininger *et al.*, 2000). Recently, echinacoside has been shown to inhibit inflammation and wound healing in rats (Speroni *et al.*, 2002). *Echinacea angustifolia* root polysaccharide fraction (EPF) effectively inhibited a croton oil-induced edema when applied topically while an intravenous injection (0.5 mg/kg) inhibited a carrageenan-induced edema (Tubaro *et al.*, 1987). The intravenous injection was slightly more effective than topical applications. Water extracts of *E. angustifolia* root (EAE) were less effective than the polysaccharide fraction at inhibiting carrageenan-induced edema. To achieve similar inhibition rates, 100 times more EAE was required compared to EPF. The application of *Echinacea* alkamides was shown to inhibit 5-lipoxygenase, a key enzyme in arachidonic acid metabolism to prostaglandins, via a competitive inhibition of the enzyme mechanism (Müller-Jakic *et al.*, 1994). Alternatively, radical scavenging activity of the highly unsaturated alkamides may reduce the oxidative reactions that may occur during arachidonic acid metabolism. Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides had the highest inhibitory activity (62.6%) against 5-lipoxygenase. However, a hexane extract of *E. angustifolia* root inhibited 81.8% of the 5-lipoxygenase activity, suggesting that additional components are involved in the enzyme inhibition (Müller-Jakic *et al.*, 1994). Alkamides (50 μg/ml) from *E. angustifolia* root also were potent cyclooxygenase (COX) inhibitors (Müller-Jakic *et al.*, 1994). Alkamides from *E. purpurea* roots were found to inhibit two COX isozymes (COX-I and COX-II) at a concentration of 100 μg/ml (Clifford *et al.*, 2002; Raso *et al.*, 2002). Alkamides inhibited the COX-I and COX-II by 36–60% and 15–46%, respectively.

B. CLINICAL EVALUATIONS

In general, the majority of the *in vitro* investigations support the biological activities of *Echinacea*. However, results from *in vivo* investigations are somewhat contradictory. More specifically, clinical investigations are less convincing due in part to the poor experimental design of the trials and the variability of *Echinacea* products tested. Virtually, all the clinical investigations prior to 1993 are flawed in some way or another. Only a few studies prior to 1993 used proper methodology such as randomized,

double-blind and placebo-controlled (RDBPC) studies (Dorn, 1989; Reitz, 1990; Schmidt *et al.*, 1990; Bräunig *et al.*, 1992; Bräunig and Knick, 1993). For further details regarding trials prior to 1993, see the reviews of Wills *et al.* (2000) and Melchart *et al.* (1994). Only clinical trials relevant to upper respiratory infections (URI) or the common cold will be highlighted below.

Hydroalcohol tincture of *E. purpurea* and *E. pallida* roots effectively reduced the duration and symptoms of URI (Bräunig *et al.*, 1992; Bräunig and Knick, 1993; Dorn *et al.*, 1997). These clinical investigations were RDBPC studies that showed that 90 drops of *E. purpurea* or *E. pallida* root tincture/day (i.e., equivalent to 900 mg root/day) shortened the duration of the URI by 3.2 and 3.9 days for bacterial and viral infections, respectively. However, daily doses of 450 mg *Echinacea* did not significantly affect URI (Bräunig *et al.*, 1992). Brinkeborn *et al.* (1999) used a four-armed RDBPC to evaluate three *Echinacea* products and a placebo as treatment for common cold. Subjects (246) were randomly placed into 1 of 4 groups and were instructed to take the preparation (2 tablets, 3 times daily) at the first onset of cold and visit a physician within 2 days of the cold onset. Group one received *E. purpurea* extract (6.78 mg, Echinaforce®—95% herb and 5% root), group two, a concentrated fresh *E. purpurea* (95% herb and 5% roots; 48.3 mg), and the third group, a root extract of *E. purpurea* (29.6 mg). The fourth group was given a placebo. Reduction in mean physician assessed symptom index from day 1 or 2 to day 7 was 29% for the placebo, 45% for group three, and 63 and 64% for groups one and two, respectively. Groups one and two's physician assessments were significantly higher than group three and the placebo. Based on the dosage, groups one, two and three had daily *Echinacea* intakes of 41,290 and 178 mg, respectively, suggesting that *Echinacea* intake was not the driving force behind the improved physician assessments observed in group one compared to group three (Brinkeborn *et al.*, 1999). The efficacy of the treatments in groups one and two, as judged by physician was approximately 68 and 78%, respectively, and by patients was 78 and 84%, respectively. This study supports the earlier report that significant reduction in clinical cold symptoms was observed when patients were given 1500 mg *E. purpurea*/day (Brinkeborn *et al.*, 1998). Again, the validity of the data can be supported by the use of RDBPC experimental design. Hoheisel *et al.* (1997) and Schulten *et al.* (2001) evaluated the pressed juice product Echinacin® under a RDBPC study to determine clinical relevance of this *Echinacea* preparation. Treatment with Echinacin® shortened the duration of the cold by 4 days, with fewer symptoms and with 20% fewer subjects developing fully expressed colds (Hoheisel *et al.*, 1997). Schulten *et al.* (2001) found that the duration of the cold was reduced by 3 days for subjects treated with Echinacin®; however, full expression of the common cold was not significantly different between the treatment group and the placebo group.

The treatment group did have significantly lower scores for rhinorrhea, nasal congestion and sore throats, suggesting that treatment with *Echinacea* could alleviate some symptoms of the common cold. Wüstenberg *et al.* (1999) reported similar results using the preparation Esberitox®, a product made with *Echinacea* root, wild indigo root and white cedar leaves.

In contrast to the positive outcomes provided before, several reports have shown that *Echinacea* does not significantly reduce URI. Schöneberger (1992) found no significant differences in the frequency (although lower) and intensity of URI between the treatment group and placebo group. This study used the pressed juice of *E. purpurea* at a dose of 8 ml/day over 8 weeks and involved 108 patients. In five randomized placebo-controlled studies involving the same 134 patients, relative phagocytic activity of PNG and leukocytes number in peripheral blood cells were enhanced in only two of the five studies (Melchart *et al.*, 1995). Of the two studies testing positive, only one used *Echinacea* as the sole source of immune-enhancing ingredient. In this study, ethanol extracts of *E. purpurea* (1000 mg) were taken orally for five days by the 134 patients. A 54% increase in phagocytosis was measured. Treatments containing various levels of ethanol extracts of *E. purpurea* root, *E. pallida* root and *E. purpurea* herb did not enhance phagocytosis or leukocytes in peripheral blood (Melchart *et al.*, 1995). Schwarz *et al.* (2002) also reported that *E. purpurea* herb did not enhance phagocytosis activity of polymorphonuclear leukocytes. A three-armed RDBPC involving 302 patients was conducted to evaluate the effects of *E. purpurea* and *E. angustifolia* root extracts on URI (Melchart *et al.*, 1998). Approximately 50 drops (91 mg) of the extracts were administered twice daily. Time to the first infections were 65, 66 and 69 days for the groups given a placebo, *E. angustifolia* root extract or *E. purpurea* root extract, respectively. The percentage of the patients infected were 37, 32 and 29% for groups given a placebo, *E. angustifolia* root extract or *E. purpurea* root extract, respectively. All data were not significantly different from the placebo groups, thus no benefit was observed (Melchart *et al.*, 1998). In an experimental rhinovirus common cold model, patients treated with 900 mg/day dose of *E. purpurea* during a period of 14 days prior to and five days after viral challenge were not significantly protected from infection (Turner *et al.*, 2000). Rhinovirus infection occurred in 44% of the subjects treated, compared to the 57% in the placebo group ($p = 0.3$). A common cold developed in 50 and 59% of subjects in the *Echinacea* and placebo groups, respectively. Grimm and Müller (1999) reported that 65 and 74% of *Echinacea* and placebo groups, respectively, had at least one cold or respiratory infection during the 8-week study. The average number of colds was 0.78 and 0.93 for the *Echinacea* and placebo groups, respectively. The duration of the colds was reduced by two days in the group

treated with the expressed juice of *Echinacea* diluted to 22% w/alcohol (8 ml/day). However, this reduction in cold duration was insignificant.

From the clinical evaluations reported to date, no clear recommendation can be drawn with regard to the effectiveness of *Echinacea* in clinical environments. However, the use of *Echinacea* as a treatment for colds or symptoms of colds are more strongly supported by clinical studies than using *Echinacea* as a prophylactic. The use of standardized extracts and dosages may be a better approach for evaluating *Echinacea* in clinical studies.

C. SAFETY/TOXICOLOGY

1. Safety of Echinacea

Echinacea enhances the immune system via nonspecific, antigen-independent mode of action (Wüstenberg *et al.*, 1999). With few exceptions, *Echinacea* has been found to be safe for consumption, with the number of adverse effects being minimal in many clinical investigations. However, the toxicological properties of *Echinacea* have not been fully explored. Only one report (Mengs *et al.*, 1991) systematically evaluated the toxicological properties of *E. purpurea*, whereas other reports are afterthoughts with regard to safety.

Mengs *et al.* (1991) used rat and mice models to assess the acute and subacute toxicities, mutagenicity and carcinogenicity. The LD_{50} in rats was $>15{,}000$ and >5000 mg *Echinacea*/kg when administered orally and intravenously, respectively. The *Echinacea* dosage needed to reach the LD_{50} in mice was even higher at concentrations $>30{,}000$ and $>10{,}000$ mg/kg for oral and intravenous administration, respectively. Subacute toxicity was determined using oral doses of *Echinacea* at 800, 2400 or 8000 mg/kg daily (Mengs *et al.*, 1991). A significant reduction in alkaline phosphatase was found in the male rats treated with 2400 and 8000 mg/kg; whereas, a significant rise in prothrombin time was found in female rats fed 2400 and 8000 mg *Echinacea*/kg. Necropsy results and histology failed to show the differences between the treated and control animals; thus no subacute toxicity was noted. No toxicity was found in the bacterial mutagenicity assays used between 8 and 5000 μg *E. purpurea*/plate. No statistical increase in mutation frequency was found in mouse lymphoma assays at concentrations up to 5000 μg/ml. In the human lymphocyte assay, mitotic inhibition was not detected at concentrations up to 5000 μg/ml; however, a significant increase in cells with aberrations was noted only at the 20 h sampling and in the 5000 μg/ml treatment. Nonsignificance was retained at the 44 h measurement. No significant increase in polychromatic erythrocytes was observed after the mice were administered a single oral dose of 25,000 mg *E. purpurea*/kg (Mengs *et al.*, 1991). The *in vitro* carcinogenicity confirmed the nontoxic effect

of other assays. See *et al.* (1997) reported that a 1000 μg/ml concentration of *Echinacea* was nontoxic based on viability of PBMC assays.

No significant differences were observed in the number of malformations, live births, or spontaneous abortions between the control group and study group (Gallo *et al.*, 2000). The study group consisted of 206 women who used *Echinacea* during pregnancy. Of the 206 women, 54% consumed *Echinacea* during the first trimester whereas only 8% consumed *Echinacea* in all three trimesters. *Echinacea* consumption varied between 250 and 1000 mg/day in tablets and from 5 to 30 drops of tincture per day. Gallo *et al.* (2000) recommended further testing on larger populations and suggested that standardized dosage may provide improved statistical power.

2. *Adverse reaction to Echinacea*

The use of *Echinacea* is not recommended for patients with autoimmune conditions, such as multiple sclerosis and AIDS, or those taking drugs to suppress immune response (e.g., corticosteroids) (Blumenthal, 1998; Gruenwald *et al.*, 2000). However, these recommendations appear to be based on speculation more than rigorous, peer-reviewed research. Research is needed to further support or refute the claim that individuals with autoimmune conditions should not use *Echinacea*.

The most notable adverse reaction to *Echinacea* was reported in Australia (Mullins, 1998, 2000). In this case, the subject orally consumed twice the recommended dose (patient consumed one teaspoon) of a 40% ethanol in water extract of *Echinacea*, which was the equivalent to 3825 mg of *E. angustifolia* (whole plant) and 150 mg dried *E. purpurea* root. Initial symptoms include burning of the mouth and throat, which is not an uncommon sensation when consuming *Echinacea*, followed by chest tightness, general urticaria and diarrhea. The subject recovered 2 h after self-administration of promethazine. A 3 mm flare was observed in a skin prick test using the same extract ingested by the subject whereas a 3 mm wheal and 5 mm flare formed in the test from a glycerin extract from the same manufacturer (Mullins, 1998). Other dietary components consumed by the subject gave negative reactions. Additional testing using radioallergosorbent (RAST) revealed *Echinacea*-binding immunoglobulin E (IgE) in the subject's serum. The patient did decline a rechallenge of the other dietary supplements taken around the time of the *Echinacea*-associated anaphylaxis. Subsequent testing of 84 subjects, with asthma or allergic rhinitis, using the above mentioned *Echinacea* products resulting in 16 (or 19%) positive responses in the skin prick test. Only two of the 16 subjects had previously consumed *Echinacea*, raising the question of potential cross-reactivity of *Echinacea* proteins and other allergens.

Further evaluation of adverse reactions to *Echinacea* has been recently reported (Mullins and Heddle, 2002). In addition to the case (i.e., case 1) given above, four additional case studies showed that *Echinacea* caused adverse reaction in subjects. An acute asthma attack occurred along with itchy and watery eyes and runny nose after the subject ingested an *Echinacea*-containing tea. The skin prick test using the same extract as in case 1 produced a 3 mm wheal. A third case involved a health professional who noted general urticaria, facial and upper airway angioedema, difficulty in swallowing and dizziness after consuming a tablet containing *Echinacea*. The skin prick test was negative to the same extract as in case 1, except that the extract was 1-year-old at the time the test was administered. However, a positive RAST score was reported. A third case involved the onset of asthma after ingestion of *Echinacea*-containing tablets. A 2 mm wheal formed after exposure to the 6-month-old extract used in case 1. The RAST test was negative for this subject. The final case involved the development of a pruritic rash after consumption of tablets containing *Echinacea*. Exposure to a 5-month-old extract, used in case 1, in the skin prick test gave negative results. In addition to these five case studies, Mullins and Heddle (2002) summarized 51 reports of adverse reaction to *Echinacea*. Twenty-six (26) of the 51 reports were possibly IgE-mediated hypersensitivity.

Other components in the *Echinacea* products may contribute to many of the adverse reactions reported, not only Australian, but also the United States, United Kingdom, Canada and New Zealand. Additional studies are needed to further support the IgE-mediated hypersensitivity reported in *Echinacea*-containing products. Studies could include growing and processing of *Echinacea* under environmentally controlled conditions, with the intent to minimize foreign contaminants, and administration of extracts to patients under supervision. Although this would not represent real-world conditions, it may further support or refute the IgE-mediated hypersensitivity observed in subjects taking commercial *Echinacea* products. Additional work is also needed to identify the component responsible for the IgE-mediated hypersensitivity.

V. *ECHINACEA* AS A FUNCTIONAL FOOD ADDITIVE

Several reports have recently been published on the *in vivo* (Sloley *et al.*, 2001—see section IV, subsection 3) and *in vitro* (Hu and Kitts, 2000; Rininger *et al.*, 2000) antioxidant activity. Hu and Kitts (2000) found that a methanol extract of *E. pallida* had a higher antioxidant activity than *E. purpurea* and *E. angustifolia*. In addition, the relationship between CAP and antioxidant activity was established. The CAP concentrations of 2.61, 1.11, and 0.49% were found in *E. pallida*, *E. angustifolia*, and *E. purpurea*,

respectively, which correlated to the antioxidant activity. Hu and Kitts (2000) hypothesized that echinacoside may account for the antioxidant activity based on the observation that the *E. purpurea* lacked echinacoside and had the lowest antioxidant potential. Many of their antioxidant assays were applicable to biological systems other than foods.

In an effort to find more efficient food antioxidants, the Food Chemistry Research Laboratory at North Dakota State University (Hall *et al.*, 2001) has preliminarily evaluated the antioxidant capacity of *Echinacea* in oil systems. The oxidative stability of sunflower oil, using Rancimat (110°C), was determined as a means to evaluate the antioxidant efficiencies of the ground herbal and root portions of *E. purpurea* and *E. angustifolia*. With the exception of *E. angustifolia* root, all antioxidant activity increased as the concentration of *Echinacea* increased from 0.05 to 1% (Figure 9). The antioxidant activity of the 0.5 and 1% addition level of *E. angustifolia* root was lower than the 0.05 and 0.1% concentrations suggesting that sufficient concentrations of other components such as metals may have contributed to the limited antioxidant activity. Up to 480 ppm of iron can be found in the taproot of *E. angustifolia* (Hobbs, 1996). The roots of *E. purpurea* is more rhizome-like than a true taproot, which may result in a lower iron content and the lack of the observed decrease in antioxidant activity as concentration increased.

A second study involved the addition of hexane and ethanol extracts of *E. purpurea* and *E. angustifolia* to stripped corn oil (50°C) and oxidation

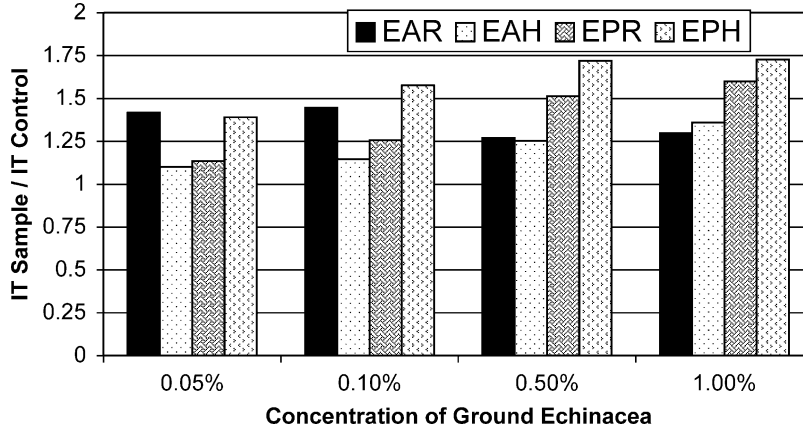

FIG. 9 Sunflower oil stability (induction time (IT) sample/IT control) under Rancimat (110°C) conditions and in the presence of ground *Echinacea* (0.05–1%): *E. purpurea* herb (EPH) and root (EPR), and *E. angustifolia* herb (EAH) and root (EAR).

assessed using peroxide value. The ethanol extracts of the *Echinacea* roots significantly inhibited the oxidation of stripped corn oil to a greater extent than the other treatments (Figure 10). The 0.5% level of the ethanol root extracts was significantly better than the 0.05% concentration at preventing oxidation. The 0.5% concentration of the ethanol extracts of the herb was less efficient than the 0.05%; however, the peroxide values for both the treatments were higher than the control and thus were pro-oxidants. All other extracts of the herbal parts of *Echinacea* promoted the oxidation (Figure 10). A positive relationship existed between phenolic content of the extracts and antioxidant activity. A qualitative assessment using HPLC showed that the CAP levels for *E. angustifolia* root were higher than other *Echinacea* products (Hall *et al.*, 2001).

In addition to oil-stability evaluations, AA retention was evaluated in orange juice treated with *E. purpurea* and *E. angustifolia* extracts and stored at room temperature. Our initial studies at North Dakota State University showed that the addition of ground *Echinacea* plant tissue was ineffective at preventing AA loss. For example, only 8% of the AA remained after 48 h in the orange juice treated with dried aerial parts. A 30% loss of AA after 48 h was found in the control juice. In contrast, 91 and 94% of AA was retained in the orange juice treated with ethanol extracts of the aerial parts and roots, respectively. In a subsequent study,

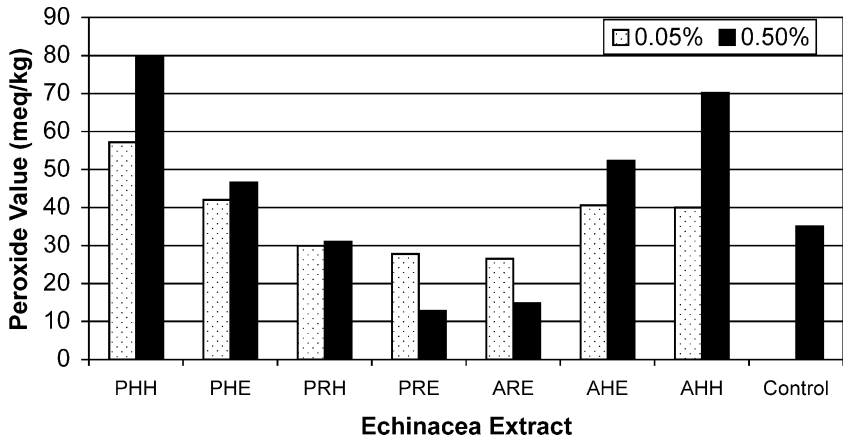

FIG. 10 Peroxide values of corn oil treated with various *Echinacea* extracts and concentration (0.05 and 0.5%). Hexane extracts of *E. purpurea* herb (PHH) and root (PRH). Ethanol extracts of *E. purpurea* herb (PHE) and root (PRE). Hexane extracts of *E. angustifolia* herb (AHH). Ethanol extracts of *E. angustifolia* herb (AHE) and root (ARE).

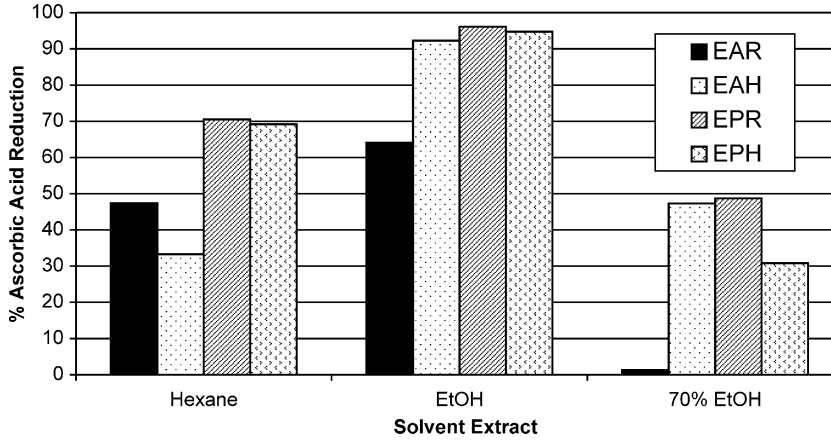

FIG. 11 Ascorbic acid reduction in orange juice treated with various *Echinacea* extracts (1%): *E. purpurea* herb (EPH) and root (EPR), and *E. angustifolia* herb (EAH) and root (EAR).

the plant materials were extracted sequentially using hexane, ethanol (95%) and ethanol:water (70:30), and the resulting extracts added separately to orange juice at 1%. At the 5-day measurement, the 70% ethanol extracts protected AA best with an average reduction in AA of 32%, compared to 55 and 87% for hexane and ethanol (95%) extracts (Figure 11). In general, the *E. angustifolia* root provided the most protection against AA loss. We are currently characterizing the phytochemical constituents in the extracts to determine if a relationship exists between phenolic content or individual phenols and AA protection. In general, the addition of *Echinacea* had a positive effect on AA retention.

VI. CONCLUSION

Echinacea has been used for centuries as a medicinal plant and has been promoted recently as an immunostimulant. Research from the last two decades has shown that *Echinacea* can enhance the immune system using *in vitro* and *in vivo* indicators. Jager *et al.* (2002) reported that dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides could cross biological barriers via passive diffusion, suggesting that the alkamides may contribute to the *in vivo* effects noted by researchers. In contrast, conflicting results have

been reported during clinical evaluations of *Echinacea*, thus there is a need to standardize formulas to truly evaluate the effectiveness of *Echinacea* in biological systems. In addition, further research is needed to establish the safety of *Echinacea* in light of the reports published on adverse reactions to *Echinacea*. Recently, the National Institutes of Health awarded the University of Iowa and Iowa State University a 5-year, $6 million grant to establish a center to investigate *Echinacea* and St. Johns Wort as dietary supplements. This level of funding indicates the seriousness to which answers are needed regarding the true benefits and risks of consuming *Echinacea*.

ACKNOWLEDGEMENTS

The author thanks Dr. Jurgen Schwarz from the University of Maryland for his assistance in translating, to English, the manuscripts written in German. The author also thanks Penny Neisen for her assistance in producing the figures and references.

REFERENCES

Barrett, B., Kiefer, D., and Rabago, D. 1999. Assessing the risks and benefits of herbal medicine: an overview of scientific evidence. *Altern. Ther. Health Med.* **5**(4), 40–49.

Bauer, R. 1996. *Echinacea* Drogen Wirkungen und Wirksubstanzen. *Z. Aerztl. Fortbild.* **90**, 111–115.

Bauer, R. 1997. Standardisierung von *Echinacea purpurea*-Preßsaft auf cichoriensäure und alkamide. *Z. Phytother.* **18**, 270–276.

Bauer, R. 1999a. Clinical investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 41–88. Birkhäuser Verlag, Basel, Switzerland.

Bauer, R. 1999b. Standardization of *Echinacea purpurea* expressed juice with reference to cichoric acid and alkamides. *J. Herbs Spices Med. Plants* **6**, 51–61.

Bauer, R. 2000. Chemistry, pharmacology, clinical applications of *Echinacea* products. *In* "Herbs, Botanicals and Teas as Functional Foods and Nutraceuticals" (G. Mazza and B.D. Oomah, eds), pp. 45–73. Technomic Publishing, Inc., Lancaster, PA.

Bauer, R. and Foster, S. 1991. Analysis of alkamides and caffeic acid derivatives from *Echinacea simulata* and *E. Paradoxa* roots. *Planta Med.* **57**, 447–449.

Bauer, R. and Remiger, P. 1989. TLC and HPLC analysis of alkamides in *Echinacea* drugs. *Planta Med.* **55**, 367–371.

Bauer, R. and Wagner, H. 1990. *Echinacea*: Ein Handbuch für Ärzte, Apotheker und andere Naturwissenschaftler. Wissenschaftliche Veragsgesellschaft, Stuttgart.

Bauer, R., Khan, I., Wray, V., and Wagner, H. 1987. Two acetylenic compounds from *Echinacea pallida* roots. *Phytochemistry* **26**, 1198–1200.

Bauer, R., Remiger, P., and Wagner, H. 1988a. Vergleichende DC-und HPLC Analyse der Herba-Drogen von *Echinacea purpurea*, *E. pallida*, und *E. angustifolia*. *Dtsch. Apoth. Ztg* **128**, 174–180.

Bauer, R., Khan, A., and Wagner, H. 1988b. TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. *Planta Med.* **54**, 426–430.

Bauer, R., Remiger, P., and Wagner, H. 1988c. Alkamides from the roots of *Echinacea purpurea*. *Phytochemistry* **27**, 2339–2342.

Bauer, R., Jurcic, K., Puhlmann, J., and Wagner, H. 1988d. Immunologische *in-vivo* und *in-vitro* untersuchungen mit *Echinacea*—Extrakten. *Arzneim.-Forsch.* **38**, 276–281.

Bauer, R., Remiger, P., and Wagner, H. 1989. Alkamides from the roots of *Echinacea angustifolia*. *Phytochemistry* **28**, 505–508.

Becker, H. and Hsieh, W. 1985. Chocoree-Saüre und deren Derivate aus *Echinacea*-Arten. *Z. Fuer Naturfuschung* **40**, 585–587 (Section C).

Becker, H., Hsieh, W., Wylde, R., Laffite, C., and Andary, C. 1982. Structure of Echinacoside. *Z. Fur Naturfuschung* **37**, 351–353 (Section C).

Bergeron, C., Livesey, J., Awang, D., Arnason, J., Rana, J., Baum, B., and Letchamo, W. 2000. A quantitative HPLC method for the quality assurance of *Echinacea* products on the North American market. *Phytochem. Anal.* **11**, 207–215.

Bergeron, C., Gafner, S., Batcha, L., and Angerhofer, C. 2002. Stabilization of caffeic acid derivatives in *Echinacea purpurea* L. glycerin extract. *J. Agric. Food Chem.* **50**, 3967–3970.

Bicchi, C., Binello, A., and Rubiolo, P. 2000. Determination of phenolic diterpene antioxidants in Rosemary (*Rosmarinus officinalis* L.) with different methods of extraction and analysis. *Phytochem. Anal.* **11**, 236–242.

Binns, S., Purgina, B., Bergeron, C., Smith, M., Ball, L., Baum, B., and Arnason, J. 2000. Light-mediated antifungal activity of *Echinacea* extracts. *Planta Med.* **66**, 241–244.

Binns, S., Baum, B., and Arnason, J. 2002a. A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). *Syst. Bot.* **27**, 610–632.

Binns, S., Livesey, J., Arnason, J., and Baum, B. 2002b. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *J. Agric. Food Chem.* **50**, 3673–3687.

Binns, S., Arnason, J., and Baum, B. 2002c. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochem. Syst. Ecol.* **30**, 837–854.

Binns, S., Hudson, J., Merali, S., and Arnason, J. 2002d. Antiviral activity of characterized extracts from *Echinacea* spp. (Heliantheae: Asteraceae) against Herpes simplex virus (HSV-I). *Planta Med.* **68**, 780–783.

Blumenthal, M. 1998. The Complete German Commission E. Monographs: Therapeutic Guide to Herbal Medicines. America Botanical Council, Austin, TX.

Bohlmann, F. and Hoffman, H. 1983. Further amides from *Echinacea purpurea*. *Phytochemistry* **22**, 1173–1175.

Bräunig, B. and Knick, E. 1993. Therapeutische Erfahrungen mit Echinacea pallidae bei Grippalen Infekten. *Naturheilpraxis* **1**, 72–75.

Bräunig, B., Dorn, M., and Knick, E. 1992. *Echinaceae purpureae* Radix: zur Stärkung der Körpereigene Abwehr bei Grippalen Infekten. *Z. Für Phytother.* **13**, 7–13.

Brinkeborn, R., Shah, D., and Degenring, F. 1999. Echinaforce® and other *Echinacea* fresh plant preparations in the treatment of the common cold. *Phytomedicine* **6**, 1–5.

Burger, R.A., Torres, A.R., Warren, R.P., Caldwell, V.D., and Hughes, B.G. 1997. *Echinacea*-induced cytokine production by human macrophages. *Int. J. Immunopharmacol.* **19**, 371–379.

Catchpole, O., Perry, N., da Silva, B., Grey, J., and Smallfield, B. 2002. Supercritical extraction of herbs I: Saw Palmetto, St. John's Wort, Kava Root and *Echinacea*. *J. Supercrit. Fluids* **22**, 129–138.

Cheminat, A., Zawatzky, R., Becher, H., and Brouillard, R. 1988. Caffeoyl conjugates from *Echinacea* species: structures and biological activity. *Phytochemistry* **27**, 2787–2794.

Classen, B., Witthohn, K., and Blaschek, W. 2000. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitaion of the β-Glucosy Yariv Reagent. *Carbohydr. Res.* **327**, 497–504.

Clifford, L., Nair, M., Rana, J., and Dewitt, D. 2002. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. *Int. J. Phytother. Pharmacol.* **3**, 249–253.

Coeugniet, E. and Elek, E. 1987. Immunomodulation with *Viscum album* and *Echinacea purpurea* extracts. *Onkologie* **10**(Suppl. 3), 27–33.

Csává, M., Borbás, A., Jánossy, L., Batta, G., and Lipták, A. 2001. Synthesis of the α-L-Araf-(1 → 2)-β-D-Galp-(1 → 6)-β-Galp-(1 → 6)-[α-L-Araf-(1 → 2)]-β-D-Galp-(1 → 6)-β-Gal hexasaccharide as a possible repeating unit of the cell-culture exudates of *Echinacea purpurea* arabinogalactan. *Carbohydr. Res.* **336**, 107–115.

Currier, N. and Miller, S. 2000. Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. *Exp. Gerontol.* **35**, 627–639.

Currier, N. and Miller, S. 2001. *Echinacea purpurea* and malatonin augment natural-killer cells in leukemic mice and prolong life span. *J. Altern. Complemen. Med.* **7**, 241–251.

Currier, N. and Miller, S. 2002. The effect of immunization with killed tumor cells, with/without feeding of *Echinacea purpurea* in an erythroleukemic mouse model. *J. Altern. Complement. Med.* **1**, 49–58.

Dietz, B. and Bauer, R. 2001. The constituents of *Echinacea atrorubens* roots and aeria. *Pharm. Biol.* **39**, 11–15.

Dorn, M. 1989. Milderung grippaler effekte durch ein pflazliches immunstimulans. *Nat. und Ganzheitsmedizin* **2**, 314–319.

Dorn, M., Knick, E., and Lewith, G. 1997. Placebo-controlled, double-blind study of *Echinacea pallidae* radix in upper respiratory infections. *Complement. Ther. Med.* **3**, 40–42.

Elsässer-Beile, U., Willenbacher, W., Bartsch, H., Gallati, H., Monting, J., and Von Kleist, S. 1996. Cytokine production in leukocyte cultures during therapy with *Echinacea* extract. *J. Clin. Lab. Anal.* **10**, 441–445.

Emmendörffer, A., Wagner, H., and Lohmann-Matthes, M.L. 1999. Clinical Investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 89–104. Birkhäuser Verlag, Basel, Switzerland.

Erhard, M., Kellner, J., Wild, J., Losch, U., and Hatiboglu, F.S. 1994. Effect of *Echinacea* aconitum, lachesis and apis extracts, their combinations on phagocytosis of human granulocytes. *PTR, Phytother. Res.* **8**, 14–17.

Facino, R.M., Carini, M., Aldini, G., Marinello, C., Arlandini, E., Franzoi, L., Colombo, M., Pietta, P., and Mauri, P. 1993. Direct characterization of caffeoyl esters with antihyaluronidase activity in crude extracts from *Echinacea angustifolia* roots by fast atom bombardment tandem mass spectrometry. *Farmaco* **48**, 1447–1461.

Facino, R.M., Carini, M., Aldini, G., Saibene, L., Pietta, P., and Mauri, P. 1995. Echinacoside and caffeoyl conjugates protect collagen from free radical-induced degradation: a potential use of *Echinacea* extracts in the prevention of skin photodamage. *Planta Med.* **61**, 510–514.

Foster, S. 1985. *Echinacea*: the purple coneflower. *Am. Hortic.* Aug., 14–17.

Foster, S. 1991. The species: identification and distribution. *In* "*Echinacea*, Nature's Immune Enhancer", pp. 107–115. Healing Arts Press, Rochester, VT.

Gahler, R., Barton, R., Slama, J., Chang, C. 2001. *Echinacea* supplement and method of manufacture. World Intellectual Property Organization, *International Publication Number* WO 01/22977.

Gaisbauer, M., Schleich, Th., Stickl, H., and Wilczek, I. 1990. Untersuchungen zum eijfluß von *Echinacea purpurea* moench auf die phagozytose von granulozyten mitels messung der chemilunineszenz. *Arzneim.-Forsch. (Drug Res.)* **40**, 594–597.

Gallo, M., Sarkar, M., Au, W., Pietrzak, K., Comas, B., Smith, M., Jaeger, T., Einarson, A., and Koren, G. 2000. Pregnancy outcome following gestational exposure to *Echinacea*. *Arch. Intern. Med.* **160**, 3141–3143.

Giger, E., Keller, F., and Baumann, T. 1989. Fructans in *Echinacea* and in its phytotherapeutic preparations. *Planta Med.* **55**, 638–639.

Glowniak, K., Zgorka, G., and Kozyra, M. 1996. Solid-phase extraction and reversed-phase high-performance liquid chromatography of free phenolic acids in some *Echinacea* species. *J. Chromatogr. A* **730**, 25–29.

Goel, V., Chang, C., Slama, J., Barton, R., Bauer, R., Gahler, R., and Basu, T. 2002. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. *Int. Immunopharmacol.* **2**, 381–387.

Goti, R., Pomponio, R., Bertucci, C., and Cavrini, V. 2002. Simultaneous analysis of the lipophilic and hydrophilic markers of *Echinacea* plant extracts by capillary electrophoresis. *J. Sep. Sci.* **25**, 1079–1086.

Gray, D., Roberts, C., Rottinghaus, G., Garrett, H., and Pallardy, S. 2001. Quantification of root chicoric acid in purple coneflower by near infrared reflectance spectroscopy. *Crop Sci.* **41**, 1159–1161.

Grimm, W. and Müller, H. 1999. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. *Am. J. Med.* **106**, 138–143.

Gruenwald, J., Brendler, T., and Jaenicke, C. 2000. *In* "PDR for Herbal Medicines", 2nd Ed., pp. 261–266. Medical Economics Company, NJ.

Hall III, C., Schwarz, J., Shultz, K., 2001. The antioxidant activity of the purple coneflower (*Echinacea*). Abstracts of the 92nd AOCS Annual Meeting & Expo, May 13–16, Minneapolis, MN. (http://www.aocs.org/archives/am2001wp.htm).

He, X., Lin, L., Bernart, M.W., and Lian, L. 1998. Analysis of alkamides in roots and achenes of *Echinacea purpurea* by liquid chromatography–electrospray mass spectrometry. *J. Chromatogr. A* **815**, 205–211.

Health Canada, 1999. *Echinacea* Root Labelling Standards. Health Canada Therapeutic Products Programme, Ottawa, Ontario.

Heyl, F. and Stanley, J. 1914. Analyses of two *Echinacea* roots. *Am. J. Pharm.* **86**, 450–455.

Hobbs, C. 1996. "*Echinacea*: The Immune Herb", pp. 31–35. Botanic Press, Santa Cruz, CA.

Hoheisel, O., Sandberg, M., Bertram, S., Bulitta, M., and Schäfer, M. 1997. *Echinacea* treatment shortens the course of the common cold: a double-blind placebo-controlled clinical trial. *Eur. J. Clin. Res.* **9**, 261–269.

Hu, C. and Kitts, D. 2000. Studies on the antioxidant activity of *Echinacea* root extract. *J. Agric. Food Chem.* **48**, 1466–1472.

Jacobson, M. 1954. Occurrence of a pungent insecticidal principle in American Coneflower roots. *Science* **120**, 1028–1029.

Jager, H., Meinel, L., Dietz, B., Lapke, C., Bauer, R., Merkle, H., and Heilmann, L. 2002. Transport of alkamides from *Echinacea* species through Caco-2 monolayers. *Planta Med.* **68**, 469–471.

Johnson, R., Sun, J.C., and Sun, J. 2002. A pervaporation-microfiltration-osmotic distillation hybrid process for concentration of ethanol–water extracts of the *Echinacea* plant. *J. Membr. Sci.* **209**, 221–232.

Kabganian, R., Carrier, D., and Sokhansanj, S. 2002. Physical characteristic and drying rate of *Echinacea* root. *Drying Technol.* **20**, 637–649.

Kim, H., Durance, T., Scaman, C., and Kitts, D. 2000a. Retention of alkamides in dried *Echinacea purpurea*. *J. Agric. Food Chem.* **48**, 4187–4192.

Kim, H., Durance, T., Scaman, C., and Kitts, D. 2000b. Retention of caffeic acid derivatives in dried *Echinacea purpurea*. *J. Agric. Food Chem.* **48**, 4182–4186.

King, P., Ma, G., Miao, W., Jia, Q., McDougall, B., Reinecke, M., Cornell, C., Kuan, J., Kim, T., and Robinson, W. 1999. Structure–activity relationships: analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. *J. Med. Chem.* **42**, 497–509.

Kreis, W., Sußner, U., and Nüsslein, B. 2000. Reinigung und Charakterisierung Einer Polyphenoloxidase aus der Arzneidroge *Echinacea purpurea* herba (Sonnenhutkraut). *J. Appl. Bot. (Angew. Bot.)* **74**, 106–112.

Laasonen, M., Harmia-Pulkkinen, T., Simard, C., Michiels, E., Rasanen, K., and Vuorela, H. 2002. Fast identification of *Echinacea purpurea* dried roots using near-infrared spectroscopy. *Anal. Chem.* **74**, 2493–2499.

Lamidey, A., Fernon, L., Pouysegu, L., Delattre, C., Quideau, S., and Pardon, P. 2002. A convenient synthesis of the *Echinacea*-derived immunostimulator and HIV-1 integrase inhibitor (−)-(2*R*, 3*R*)-chicoric acid. *Helv. Chim. Acta* **85**, 2328–2334.

Lersch, C., Zeuner, M., Bauer, A., Siebenrock, K., Hart, R., Wagner, F., Fink, U., Dancygier, H., and Classen, M. 1990. Stimulation of the immune response in outpatients with hepatocellular carcinomas by low doses of cyclophosphamide (LDCY), *Echinacea purpurea* extracts (Echinacin®) and thymostimunlin. *Arch. Geschwulstforsch.* **60**, 379–383.

Lersch, C., Zeuner, M., Bauer, A., Siemens, M., Hart, R., Drescher, M., Fink, U., Dancygier, H., and Classen, M. 1992. Nonspecific immunostimulation with low doses of cyclophosphamide (LDCY), Thymnostimulin, *Echinacea purpurea* extracts (Echinacin®) in patients with far advanced colorectal cancers: preliminary results. *Cancer Investig.* **10**, 343–348.

Li, T.S.C. 1998. *Echinacea*: cultivation and medicinal value. *HortTechnology* **8**, 122–129.

Li, T.S.C. and Wardle, D. 2001. Effects of root drying temperature and moisture content on the levels of active ingredients in *Echinacea roots*. *J. Herbs Spices Med. Plants* **8**, 15–22.

Lienert, D., Anklam, E., and Panne, U. 1998. Gas chromatography–mass spectral analysis of roots of *Echinacea* species and classification by multivariate data analysis. *Phytochem. Anal.* **9**, 88–98.

Livesey, J., Awang, D., Arnason, J., Letchamo, W., Barrett, M., and Pennyroyal, G. 1999. Effect of temperature on stability of marker constituents in *Echinacea purpurea* root formulations. *Phytomedicine* **6**, 347–349.

Luettig, B., Steinmüller, C., Gifford, G., Wagner, H., and Lohmann-Matthes, M. 1989. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. *J. Natl Cancer Inst.* **81**, 669–675.

Luo, X., Chen, B., Yao, S., and Zeng, J. 2003. Simultaneous analysis of caffeic acid derivatives and alkamides in roots and extracts of *Echinacea purpurea* by high performance liquid chromatography-photodiode array detection-electrospray mass spectrometry. *J. Chromatogr. A* **986**, 73–81.

McDougall, B., King, P., Wu, B., Hostomsky, Z., Reinecke, M., and Robinson, W. 1998. Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob. Agents Chemother.* **42**, 140–146.

Melchart, D. and Linde, K. 1999. Clinical investigations of *Echinacea* phytopharmaceuticals. *In* "Immunomodulatory Agents from Plants" (H. Wagner, ed.), pp. 105–118. Birkhäuser Verlag, Basel, Switzerland.

Melchart, D., Linde, K., Worku, F., Bauer, R., and Wagner, H. 1994. Immunomodulation with *Echinacea*—a systematic review of controlled clinical trials. *Phytomedicine* **1**, 245–254.

Melchart, D., Linde, K., Worku, F., Sarkady, L., Holzmann, M., Jurcic, K., and Wagner, H. 1995. Results of five randomized studies on the immunomodulatory activty of preparations of *Echinacea*. *J. Altern. Complement. Med.* **1**, 145–160.

Melchart, D., Walther, E., Linde, K., Brandmaier, R., and Lersch, C. 1998. *Echinacea* roots extracts for the prevention of upper respiratory tract infections: a double-blind, placebo-controlled randomized trial. *Arch. Fam. Med.* **7**, 541–545.

Melchart, D., Clemm, C., Weber, B., Draczynski, T., Worku, F., Linde, K., Weidenhammer, W., Wagner, H., and Saller, R. 2002. Polysaccharide isolated from *Echinacea purpurea* herba cell cultures to counteract undesired effects of chemotherapy—a pilot study. *Phytother. Res.* **16**, 138–142.

Mengs, U., Clare, C., and Poiley, J. 1991. Toxicity of *Echinacea purpurea*. Acute, subacute and genotoxicity studies. *Arzneim.-Forsch. (Drug Res.)* **41**, 1076–1081.

Müller-Jakic, B., Breu, W., Probstle, A., Redl, K., Greger, H., and Bauer, R. 1994. *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from *Echinacea* and *Achillea* species. *Planta Med.* **60**, 37–40.

Mullins, R. 1998. *Echinacea*-associated anaphylaxis. *Med. J. Aust.* **168**, 170–171.

Mullins, R. 2000. Allergic reactions to *Echinacea*. *J. Allergy Clin. Immunol.* **105**, S340–S341.

Mullins, R. and Heddle, R. 2002. Adverse reactions associated with *Echinacea*: the Australian experience. *Ann. Allergy Asthma Immun.* **88**, 42–51.

Nüsslein, B., Kurzmann, M., Bauer, R., and Kreis, W. 2000. Enzymatic degradation of cichoric acid in *Echinacea purpurea* preparations. *J. Nat. Prod.* **63**, 1615–1618.

Osowski, S., Rostock, M., Bartsch, H., and Massing, U. 2000. Zur Pharmazeutischen Vergleichbarkeit von Therapeutisch Verwendeten Echinacea-Praparaten. *Forschende. Komplementearmedizin Klassische Naturheilkunde (Res. Complement. Nat. Classical Med.)* **7**, 294–300.

Perry, N.B., Van Klink, J.W., Burgess, E.J., and Parmenter, G.A. 1997. Alkamide levels in *Echinacea purpurea*: a rapid analytical method revealing differences among roots, rhizomes, stems, leaves and flowers. *Planta Med.* **63**, 58–62.

Perry, N., Van Klink, J.W., Burgess, E.J., and Parmenter, G.A. 2000. Alkamide levels in *Echinacea purpurea*: effects of processing, drying, storage. *Planta Med.* **66**, 54–56.

Perry, N., Burgess, E., and Glennie, V. 2001. *Echinacea* standardization: analytical methods for phenolic compounds and typical levels in medicinal species. *J. Agric. Food Chem.* **49**, 1702–1706.

Pietta, P., Mauri, P., and Bauer, R. 1998a. MEKC analysis of different *Echinacea* species. *Planta Med.* **64**, 649–652.

Pomponio, R., Gotti, R., Hudaib, M., and Cavrini, V. 2002. Analysis of phenolic acids by micellar electrokinetic chromatography: application of *Echinacea purpurea* plant extracts. *J. Chromatogr. A* **945**, 239–247.

Proksch, A. and Wagner, H. 1987. Structural analysis of a 4-*O*-methyl-glucuronoarabinoxylan with immuno-stimulating activity from *Echinacea purpurea*. *Phytochemistry* **26**, 1989–1993.

Raso, G., Pacilio, M., Di-Carlo, G., Esposito, E., Pinto, L., and Meli, R. 2002. *In-vivo* and *in-vitro* anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*. *J. Pharm. Pharamacol.* **54**, 1379–1383.

Rehman, J., Dillow, J.M., Carter, S.M., Chou, J., Le, B., and Maisel, A.S. 1999. Increased production of antigen-specific immunoglobulins G and M following *in vivo* treatment with the medicinal plants *Echinacea angustifolia* and *Hydrastis canadensis*. *Immunol. Lett.* **68**, 391–395.

Reich, E., Blatter, A., Jorns, R., Kreuter, M., and Thiekotter, K. 2002. An AOAC peer-verified method for identification of *Echinacea* species by HPTLC. *J. Planar Chromatogr.* **15**, 244–251.

Reinke, R., King, P., Victoria, J., McDougall, B., Ma, G., Mao, Y., Reinecke, M., and Robinson, W. 2002. Dicaffeoyltartaric acid analogues inhibit human immunodeficiency virus type 1 (HIV-1) integrase and HIV-1 replication at nontoxic concentrations. *J. Med. Chem.* **45**, 3669–3683.

Reitz, H. 1990. Immunomodulation mit Pflanzlichen Wirkstoffen: Eine Wissenschaftliche Studie am Beispiel Esberitox N. *Notabene Med.* **20**, 362–366.

Rininger, J., Kickner, S., Chigurupati, P., McLean, A., and Franck, Z. 2000. Immunopharmacological activity of *Echinacea* preparations following simulated digestion of murine macrophages and human peripheral blood mononuclear cells. *J. Leukoc. Biol.* **68**, 503–510.

Robinson, W., Reinecke, M., Abdel-Malek, S., Jia, Q., and Chow, S. 1996a. Inhibitors of HIV-1 replication that inhibit HIV integrase. *Proc. Natl Acad. Sci. USA* **93**, 6326–6331.

Robinson, W., Cordeiro, M., Abdel-Malek, S., Jia, Q., Chow, S., Reinecke, M., and Mitchell, W. 1996b. Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: inhibition of the core catalytic domain of human immunodeficiency virus integrase. *Mol. Pharmacol.* **50**, 846–855.

Roesler, J., Emmendörffer, A., Steinmüller, C., Luettig, B., Wagner, H., and Lohmann-Matthes, M. 1991a. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of phagocyte system. *Int. J. Immunopharmacol.* **13**, 931–941.

Roesler, J., Steinmüller, C., Kiderlen, A., Emmendörffer, A., Wagner, H., and Lohmann-Matthes, M. 1991b. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with *Listeria monocytogenes* and *Candida albicans*. *Int. J. Immunopharmacol.* **13**, 27–37.

Rogers, K.L., Grice, I.D., Mitchell, C.J., and Griffiths, L.R. 1998. High performance liquid chromatography determined alkamide levels in Australian-grown *Echinacea* spp. *Aust. J. Exp. Agric.* **38**, 403–408.

Schieffer, G. 2000. Validated HPLC method for caffeic-acid derivatives and alkylamides of *Echinacea* solid-dosage forms using a single extraction procedure. *J. Am. Nutraceutical Assoc.* **3**, 67–81.

Schieffer, G. and Kohn, M. 2002. HPLC assay of *Echinacea purpurea*/Goldenseal (*Hydrastis canadensis*) combination formulations for the phenolic acids, alkylamides, alkaloids. *J. Liq. Chromatogr. Rel. Technol.* **25**, 263–274.

Schmidt, U., Albrecht, M., and Schenk, N. 1990. Immunstimulans Senkt Häufigkeit Grippaler Infekt. Plazebokontrollierte Doppelblindstudie mit 646 Studenten der Kölner Universität. *Nat. Und Ganzheitsmedizin* **3**, 277–281.

Schulten, B., Bulitta, M., Ballering-Bühl, B., Köster, U., and Schäfer, M. 2001. Efficay of *Echinacea purpurea* in patients with a common cold. *Arzneim.-Forsch. (Drug Res.)* **51**, 563–568.

Schulthess, B.H., Giger, E., and Baumann, T.W. 1991. *Echinacea*: anatomy, phytochemical pattern, germination of the achene. *Planta Med.* **57**, 384–388.

Schulz, H., Pfeffer, S., Quilitzsch, R., Steuer, B., and Reif, K. 2002. Rapid and non-destructive determination of the echinacoside content in *Echinacea* roots by ATR-IR and NIR spectroscopy. *Planta Med.* **68**, 926–929.

Schumacher, A. and Friedberg, K. 1991. Untersuchungen Zur Wirkung von Echinacea angustifolia auf die Unspezifische Zelluläre Immunantwort der Maus. *Arzneim.-Forsch. (Drug Res.)* **41**, 141–147.

Schöneberger, D. 1992. The influence of immune stimulating effects of pressed juice from *Echinacea purpurea* on the course and severity of colds. *Forum Immunol.* **8**, 2–12.

Schwarz, E., Metzler, J., Diedrich, J., Freudenstein, J., Bode, C., and Bode, J. 2002. Oral administration of freshly expressed juice of *Echinacea purpurea* herbs fail to stimulate the nonspecific immune response in healthy young men: results of a double-blind, placebo-controlled crossover study. *J. Immunother.* **25**, 413–420.

See, D.M., Broumand, N., Sahl, L., and Tilles, J.G. 1997. *In vitro* effects of *Echinacea* and Ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. *Immunopharmacology* **35**, 229–235.

Sloley, B.D., Urichuk, L., Tywin, C., Coutts, R., Pang, P., and Shan, J. 2001. Comparison of chemical components and antioxidant capacity of different *Echinacea* species. *J. Pharm. Pharmacol.* **53**, 849–857.

South, E. and Exon, J. 2001. Multiple immune functions in rats fed *Echinacea* extracts. *Immunopharm. Immunotoxicol.* **23**, 411–421.

Speroni, E., Govoni, P., Guizzardi, S., Renzulli, C., and Guerra, M. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida Nutt.* rot extract. *J. Ethnopharmacol.* **79**, 265–272.

Steinmüller, C., Roesler, J., Grottrup, E., Franke, G., Wagner, H., and Lohmann-Matthes, M.L. 1993. Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. *Int. J. Immunopharmacol.* **15**, 605–614.

Stotzem, C., Hungerland, U., and Mengs, U. 1992. Influence of *Echinacea purpurea* on the phagocytosis of human granulocytes. *Med. Sci. Res.* **20**, 719–720.

Stuart, D.L. and Wills, R.B.H. 2000a. Alkylamides and cichoric acid levels in plant sections of *Echinacea purpurea* during growth. *J. Herbs Spices Med. Plants* **7**, 91–101.

Stuart, D.L. and Wills, R.B.H. 2000b. Factors affecting the extraction of alkylamides and cichoric acid during ethanolic processing of *Echinacea purpurea* (L.) Moench. *Aust. J. Exp. Agric.* **40**, 873–877.

Sun, L., Currier, N., and Miller, S. 1999. The American Coneflower: a prophylactic role involving nonspecific immunity. *J. Altern. Complement. Med.* **5**, 437–446.

Sun, L., Rezaei, K., Temelli, F., and Ooraikul, B. 2002. Supercritical fluid extraction of alkylamides from *Echinacea angustifolia*. *J. Agric. Food Chem.* **50**, 3947–3953.

Tubaro, A., Tragni, E., Del Negro, P., Galli, C., and Loggia, R. 1987. Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. *J. Pharm. Pharmacol.* **39**, 567–569.

Turner, R., Riker, D., and Gangemi, J.D. 2000. Ineffectiveness of *Echinacea* for prevention of experimental rhinovirus colds. *Antimicrob. Agents Chemother.* **44**, 1708–1709.

Vömel, T. 1985. Der einflußeines pflanzlichen immunostimulans auf die phagozytose von erythrozyten durch das retikulohistozytäre stystem der isoliert perfundierten rattenleber. *Arzneim.-Forsch (Drug Res.)* **35**, 1437–1439.

Wacker, A. and Hilbig, W. 1978. Virus-inhibition by *Echinacea purpurea* (Author's Transl). *Planta Med.* **33**, 89–102.

Wagner, H. and Jurcic, K. 1991. Immunologic studies of plant combination preparations. *In-vitro* and *in-vivo* studies on the stimulation of phagocytosis. *Arzneim.-Forsch.* **41**, 1072–1076.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Heur, Y.H. 1984. Immunostimulant action of polysaccharides (heteroglycans) from higher plants. Preliminary communication. *Arzneim.-Forsch.* **34**, 659–661.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Fang, J. 1985. Immunostimulating action of polysaccharides (heteroglycans) from higher plants. *Arzneim.-Forsch.* **35**, 1069–1075.

Wagner, H., Stuppner, H., Schafer, W., and Zenk, M. 1988. Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. *Phytochemistry* **27**, 119–126.

Wildfeuer, A. and Mayerhoer, D. 1994. *Echinacea Purpurea* und Eleutherococcus senticosus. *Arzneim.-Forsch. (Drug Res.)* **44**, 361–366.

Wills, R.B.H. and Stuart, D.L. 1998. Levels of active constituents in manufactured *Echinacea* products. *Chem. Aust.* **65**, 17–19.

Wills, R.B.H. and Stuart, D.L. 1999. Alkylamide and cichoric acid levels in *Echinacea purpurea* grown in Australia. *Food Chem.* **67**, 385–388.

Wills, R.B.H. and Stuart, D.L. 2000. Effect of handling and storage on alkylamide and cichoric acid levels in *Echinacea purpurea*. *J. Sci. Food Agric.* **80**, 1402–1406.

Wills, R.B.H., Bone, K., and Morgan, M. 2000. Herbal products: active constituents, modes of action and quality control. *Nutr. Res. Rev.* **13**, 47–77.

Wilson, D. 1998. Nutraceuticals: a natural choice for grain-based products. *Cereal Foods World* **43**, 718–719.

Wüstenberg, P., Köhler, G., and Stammwitz, U. 1999. Efficacy and mode of action of an immunomodulator herbal preparation containing *Echinacea*, wild indigo, white cedar. *Adv. Ther.* **16**, 51–70.