Echinacea

The genus *Echinacea*

Edited by Sandra Carol Miller

Assistant Editor — He-ci Yu

Medicinal and Aromatic Plants — Industrial Profiles

CRC PRESS

Echinacea

The genus *Echinacea*

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Echinacea

The genus *Echinacea*

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Series Preface

There is increasing interest in industry, academia, and the health sciences in medicinal and aromatic plants. In passing from plant production to the eventual product used by the public, many sciences are involved. This series brings together information that is currently scattered through an ever increasing number of journals. Each volume gives an in-depth look at one plant genus, about which an area specialist has assembled information ranging from the production of the plant to market trends and quality control.

Many industries are involved such as forestry, agriculture, chemical food, flavor, beverage, pharmaceutical, cosmetic, and fragrance. The plant raw materials are roots, rhizomes, bulbs, leaves, stems, barks, wood, flowers, fruits, and seeds. These yield gums, resins, essential (volatile) oils, fixed oils, waxes, juices, extracts, and spices for medicinal and aromatic purposes. All these commodities are traded worldwide. A dealer's market report for an item may say "Drought in the country of origin has forced up prices."

Natural products do not mean safe products and account of this has to be taken by the above industries, which are subject to regulation. For example, a number of plants that are approved for use in medicine must not be used in cosmetic products.

The assessment of safe to use starts with the harvested plant material, which has to comply with an official monograph. This may require absence of, or prescribed limits of, radioactive material, heavy metals. aflatoxic, pesticide residue, as well as the required level of active principle. This analytical control is costly and tends to exclude small batches of plant material. Large-scale contracted mechanized cultivation with designated seed or plantlets is now preferable.

Today, plant selection is not only for the yield of active principle, but for the plant's ability to overcome disease, climatic stress, and the hazards caused by mankind. Such methods as *in vitro* fertilization, meristem cultures, and somatic embryogenesis are used. The transfer of sections of DNA is giving rise to controversy in the case of some end-uses of the plant material.

Some suppliers of plant raw material are now able to certify that they are supplying organically farmed medicinal plants, herbs, and spices. The European Union directive (CVO/EU No. 2092/91) details the specifications for the *obligatory* quality controls to be carried out at all stages of production and processing of organic products.

Fascinating plant folklore and ethnopharmacology leads to medicinal potential. Examples are the muscle relaxants based on the arrow poison, curare, from species of *Chondrodendron*, and the anti-malarials derived from species of *Cinbona* and *Artemisia*. The methods of detection of pharmacological activity have become increasingly reliable and specific, frequently involving enzymes in bioassays and avoiding the use of laboratory animals. By using bioassay-linked fractionation of crude plant juices or extracts, compounds can be specifically targeted that, for example, inhibit blood platelet aggregation, or have anti-tumor, anti-viral, or any other required activity. With the assistance of robone devices all the members of a genus may be readily screened. However, the plant material must be fully authenticated by a specialist.

The medicinal traditions of ancient civilizations such as those of China and India have a large armamenoaria of plants in their pharmacopoeias that are used throughout southeast Asia. A similar situation exists in Africa and South America. Thus, a very high percentage of the world's population relies on medicinal and aromatic plants for their medicine. Western medicine is also responding. Already in Germany all medical practitioners have to pass an examination in phytotherapy before being allowed to practice. It is noticeable that throughout Europe and the U.S.A., medical, pharmacy, and health-related schools are increasingly offering training in phytotherapy.

Multinational pharmaceutical companies have become less enamored of the single compound magic bullet cure. The high costs of such ventures and the endless competition from "me too" compounds from rival companies often discourage the attemp. Independent phytomedicine companies have been very strong in Germany. However, by the end of 1995, eleven (almost all) had been acquired by the multinational pharmaceutical firms, acknowledging the lay public's growing demand for phytomedicines in the Western World.

The business of dietary supplements in the western world has expanded from the health store to the pharmacy. Alternative medicine includes plant-based products. Appropriate measure to ensure the quality, safety, and efficacy of these either already exist or are being answered by greater legislative control by such bodies as the Food and Drug Administration of the U.S.A. and the European Agency for the Evaluation of Medicinal Products, based in London.

In the U.S.A., the Dietary Supplement and Health Education Act of 1994 recognized the class of phytotherapeutic agents derived from medicinal and aromatic plants. Furthermore, under public pressure, the U.S. Congress set up an Office of Alternative Medicine and this office in 1994 assisted the filing of several investigational new drug (IND) applications, required for clinical trials of some Chinese herbal preparations. The significance of these applications was that each Chinese preparation involved several plants and yet was handled as a *single* IND. A demonstration of the contribution to efficacy of *each* ingredient of *each* plant was not required. This was a major step forward toward more sensible regulations in regard to phytomedicines.

My thanks are due to the staffs of CRC Press who have made this series possible and especially to the volume editors and their chapter contributors for the authoritative information.

Roland Hardman

Preface

We have brought together, in the preparation of this volume, studies from experts from around the world with respect to the analysis of, and potential uses for, the various species of genus *Echinacea*. During the last 20 years, there has been a virtual explosion in the uses of herbs, particularly *Echinacea*, primarily for its numerous medicinal properties, thereby rendering *Echinacea* one of the top-selling herbs of all time. Several species of the genus, i.e., *E. purpurea*, *E. angustifolia*, and *E. pallida*, have attracted considerable interest for their reported health benefits, including amelioration of several pathologies such as virus-mediated, bacterial and parasitic afflictions, and inflammatory conditions of assorted etiologies. In spite of *Echinacea* having contributed in large measure to the recent growth of the natural health products industry, there is still a lack of government regulation over this and other herb-based products. When requested to do so, many manufacturers supply evidence, albeit meager, to support their claims of the health benefits of *Echinacea*; however, much evidence is anecdotal or based on case studies. Consequently, it is not difficult to understand the hesitancy of governments to sanction these products. In other words, unlike pharmaceuticals, with their well-established, tried and true claims for potency and contraindications, there is very little hard science behind the medicinal claims for nutriceuticals and phytocompounds such as those in *Echinacea*.

The chemical composition of any herb is complex compared to any drug, the latter containing a single, known, active ingredient. The method of extraction from plants of any biologically active ingredient has a major impact on the composition of the final product. Indeed, it is the high variability in the content of active ingredients that has resulted in a need for standardization, yet this is only possible when certain prerequisites have been met: at least one well-defined ingredient has been isolated, identified, and proven to be biologically active, and the standardization procedures have not altered the composition of that ingredient to render it inactive. The "active ingredients" in various species of the genus need to be analyzed and assimilated. Several chapters in the first half of this volume deal with the taxonomy, genetics, cultivation and culture methods, and chemistry of *Echinacea*, while other chapters are concerned with the analytical evaluation of the various plant parts, including roots, stems, leaves, and flowers.

Although *Echinacea* was used in medicine by early physicians at least 200 years ago, formal use of the herb was never approved by governments and/or the conventional medical establishment, because rigorous experimental evidence did not exist for the claims of its healing properties. Moreover, with the advent of antibiotics, the medicinal potential of *Echinacea* was virtually forgotten. Subsequently, however, techniques for measuring the functional responses of different cells *in vitro* led to the rediscovery of *Echinacea*. Thus, species from the genus have recently been the subject of considerable scientific scrutiny and during the past 20 years, much effort has been aimed at dissecting out from this herb the many chemical compounds that act on cells, especially those involved in the disease-defense process. Students in the health sciences in many North American universities, and some in Europe, are now required to have some formal instruction in the use of herbal medicines. However, individuals in the various healthcare professions should be aware of the fact that not all *Echinacea* products in the marketplace are of high quality and potency. While it is generally assigned immune enhancement activities, the effectiveness of *Echinacea* in reducing/preventing the duration/severity of disease is highly dependent not only on the species of *Echinacea*, but the part of the plant used (root, stem, leaves), the age of the plant, its location, and finally, the method of extraction of the active ingredients, once they are identified. Moreover, in spite of its popularity, there is a dearth of basic, *in vivo* data indicating important parameters such

as tissue retention/distribution, blood clearance time, excretion rates, and long-term effects of this herb. Nevertheless, there is, indeed, continuously accumulating experimental evidence, revealing that plant products of genus *Echinacea* may soon see their debut alongside conventional forms of therapy, even in cancer treatment. For example, certain cells in the presence of whole extract of *Echinacea* or its derivatives are stimulated to produce a cascade of growth factors that are known to be valuable in hemopoiesis stimulation after a bone marrow transplant. Several chapters in the second half of this volume are concerned with the medicinal value of *Echinacea* and its derivatives.

In summary, the proposed volume is timely and comprehensive, and bridges the gap between the abundant molecular cataloguings of the phytochemicals present in genus *Echinacea* and the functional potential of this plant. Thus, we have strived to link botanical biochemistry with medicine in defining with solid science specific, medicinal roles for *Echinacea* in disease prevention and abatement.

We wish to thank Lisbet Skogberg for her time and talent in preparing the cover illustration, a flowering *Echinacea* plant.

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Section I

The Genus Echinacea: *Taxonomy and Genetics*

1 Taxonomic History and Revision of the Genus *Echinacea*

Sharon E. Binns, John T. Arnason, and Bernard R. Baum

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INTRODUCTION

Echinacea (Asteraceae) is a North American plant genus found in natural populations east of the Rocky Mountains in the Atlantic drainage region of the United States and Canada (Binns et al., 2002a). From snakebites to cancers, and toothaches to the common cold, medicinal preparations from plant parts of *Echinacea* species are used worldwide for their healing properties. Numerous medicinal uses of *Echinacea* were practiced historically by First Nations groups prior to their first documented uses by Western medical doctors and herbalists (Gilmore, 1911, 1913; Hart, 1981; Kindscher, 1989; Shemluck, 1982). Then, over the course of the 20th century, medical advances such as antibiotics eclipsed the prevalent use of *Echinacea* herbal medicines for infections (Hobbs, 1994).

In market surveys, *Echinacea* is consistently one of the top 10 species of herbs sold (Brevoort, 1998). This constitutes a revival in *Echinacea* use from wild and cultivated sources that is due, in part, to the hundreds of pharmacological and clinical studies conducted since the early 1980s. At present, natural health products derived from *Echinacea* plant materials are leaders in a trend toward preventive health care and alternatives to synthetic pharmaceuticals. To this end, some requirements that remain to be defined and met are accurate botanical characterization and quality control of plant materials. Without these, there can be no successful modernization of *Echinacea* and other herbal medicines to fit state-of-the-art systems of health care, knowledge, and safety. One reason for the significant lack of well-characterized plant material is the lack of clarity in taxonomic classification and identification of *Echinacea*.

In this chapter, the history and current challenges to accurate botanical identification of different *Echinacea* species and varieties are discussed. A revised taxonomy of the genus *Echinacea* will be revealed and placed reasonably within its historical nomenclatural context. Also, potential implications of this new taxonomic system will be explained, with simple suggestions for the most effective application of this information to the many users of *Echinacea* plants and medicines derived from them.

HISTORICAL TAXONOMY AND NOMENCLATURE

The name *Echinacea* is applied to an entire genus, classified within the Asteraceae (or composite family), one of the largest families of flowering plants (Hitchcock and Cronquist, 1973). European explorers in the 18th century described and named the first species in this genus, *Rudbeckia purpurea* L. (Linnaeus, 1753) from the forests of southeastern North America. At the level of the entire genus, the historical use of *Rudbeckia*, *Echinacea* Moench (1794), and a third name, *Brauneria* Necker (1790), for the same taxon were recently explained at length (Binns et al., 2001b, 2002a).

In 1818, Thomas Nuttall was the first to distinguish two different groups in the taxon, but he found them sufficiently similar so as to be varieties and not separate species. Nuttall named his newly found variety *Rudbeckia purpurea* var. *serotina* (Nuttall, 1818). This same variety was elevated to species *Echinacea serotina* (Nutt.) DC. by Candolle (1836), who described a total of four species in the genus *Echinacea*. We recently discovered a discrepancy between the current use of the name *Echinacea purpurea* (L.) Moench and the Linnaean type specimen. In short, the type specimen designated by McGregor (1968) for the taxon *E. purpurea* (L.) Moench did not match the original description by Linnaeus (1753), but it did match Candolle's description and type with the name *E. serotina* (Nutt.) DC. This error apparently occurred when Boynton and Beadle (in Small, 1903) mistakenly applied the species name *Brauneria purpurea* to what should have been called *Echinacea serotina* (their error was in the species epithet, not the use of the genus name *Brauneria*, which was later invalidated). As a result of their misinterpretation of earlier taxonomies and the error noted above, Boynton and Beadle considered the true *E. purpurea* (L.) Moench (by Linnaeus and Candolle) to be a new species, and they gave it a new name, *Brauneria laevigata*. Blake adopted this taxon in 1929 with the name *Echinacea laevigata* (Boynton & Beadle) Blake. We have proposed the conservation of the names in current use to avoid confusion in the horticultural and pharmaceutical trade (Binns et al., 2001a, 2001b). A summary of historical events pertaining to nomenclatural confusion with respect to the two taxa, called "*E. purpurea*" and "*E. laevigata*" today is found in Table 1.1.

Over the course of nearly 2 centuries, taxonomists recorded names and descriptions of a number of infrageneric taxa in the genus *Echinacea* in the floristic literature of North America. However, especially since most publications treated only various parts of the entire geographical range for *Echinacea,* they differed in the number of taxa (from 2 to 11 different groups) in various nomenclatural combinations of species and varieties (Binns, 2001). Cronquist (1945) described four species (and one variety) in a thorough taxonomic treatment, which was based on type specimens and herbarium collections. Finally, in 1968, McGregor made a significant contribution to our understanding of the genus. He sampled many wild populations and performed garden experiments for 15 years before he published his taxonomy of the genus *Echinacea*, the first treatment to cover the entire reported geographical range. McGregor (1968) recognized nine species and four varieties using morphological traits and chromosome numbers. His findings were limited by the methodology of the time and he proposed that there may be extensive genetic variation within certain wild populations of a single species or variety, and that further genetic studies were necessary. Recent hypotheses indicate that active speciation and evolution within the genus is most likely occurring in the Ozark Mountains of Missouri and Arkansas, extending into southeastern Oklahoma (Binns et al., 2002a; McKeown, 1999). This region of the Great Plains is the center of *Echinacea* diversity.

Morphologically, there is such variability within populations that users of McGregor's identification keys have struggled to definitively identify wild and cultivated *Echinacea* plant materials. For example, *E. pallida* (Nutt.) Nutt. var. *pallida* [*E. pallida* (Nutt.) Nutt.] has long been cultivated in Germany, but it was being sold as *E. pallida* (Nutt.) Nutt. var. *angustifolia* (DC.) Cronq. [*E. angustifolia* DC. var. *angustifolia*] until identification by phytochemical methods was refined in the early 1990s (Bauer and Wagner, 1991). (Taxonomic names are those from the revision [Binns et al., 2002a]. At first reference, each name is followed by square brackets with the synonym according to McGregor [1968], which is currently the one in commercial use. Revised nomenclature is used in the figures and tables.) Despite these chemotaxonomic markers for the commercial

TABLE 1.1
Summary of nomenclatural history of *Echinacea purpurea* (L.) Moench and *E. laevigata* (Boynton & Beadle) Blake since 1753

1753	Linnaeus described *Rudbeckia purpurea* using the exact phrase name of Gronovius (1739) (listed specimen no. 417 [now in Clayton Herb., BM]), and cited phrase names by Plukenet (1696), Morison (1699), and Catesby (1743).
1790	Necker described the genus *Brauneria* (no species mentioned).
1794	Moench described *Echinacea purpurea* L. with the new combination *E. purpurea* (L.) Moench.
1818	Nuttall described new taxon *Rudbeckia purpurea* L. var. *serotina*.
1821	*Rudbeckia speciosa* Link. was described (different from *R. speciosa* Wender or Shrad).
1823	Sweet described *Rudbeckia serotina* (Nutt.) Sweet.
1824	J.C. von Hoffmannsegg described *Rudbeckia hispida*.
1836	Candolle made the combination *Echinacea serotina* (Nutt.) DC. and listed the following synonyms: *R. purpurea* L. var. *serotina* Nutt.; *R. serotina* (Nutt.) Sweet; *R. speciosa* Link.; *R. hispida* Hoffmannsegg. Candolle also recognized *E. purpurea* L. Moench as a distinct taxon (description matching Linnaeus).
1836-1894	Authors acknowledged only *E. purpurea* (L.) Moench but not *E. serotina* (Nutt.) DC., such as Darby (1860) and Chapman (1889).
1894	*Brauneria purpurea* (L.) Necker ex. Porter & Britton (includes no description).
1903	Small recognized *Brauneria laevigata* Boynton & Beadle and *Brauneria purpurea* (L.) Britton as two distinct taxa.
1929	Blake made the combination *Echinacea laevigata* (Boynton & Beadle) Blake.
1945	Cronquist reduced *E. laevigata* to a variety, *E. purpurea* Moench var. *laevigata* (Boynton & Beadle) Cronq. He also created the new name *E. purpurea* Moench var. *purpurea* Cronq., synonymous with *E. purpurea* (L.) Moench (in current use, lectotypified by McGregor [1968]).
1959	*Echinacea* was accepted as first validly published name for the genus and *Brauneria* was rejected as an invalid name (Lanjouw, 1959).
2001	Binns et al. (2001b) discovered a widespread misapplication of the name *E. purpurea* (L.) Moench for the taxon that was correctly named *E. serotina* (Nutt.) DC. in 1836 and the subsequent use of *E. laevigata* (Boynton & Beadle) Blake to describe the taxon first named *Rudbeckia purpurea* L. Binns et al. (2001a) proposed conservation of the names in current use.

Source: Modified from Binns, S.E., The taxonomy, phytochemistry and biological activity of the genus *Echinacea* (Asteraceae), Ph.D. Thesis, University of Ottawa, 2001. With permission.

species, a large-scale taxonomic revision was necessary for accurate botanical identification of all the different *Echinacea* taxa, not only those sold as phytomedicines.

TAXONOMIC REVISION OF *ECHINACEA*

We sampled wild populations of each of McGregor's (1968) *Echinacea* taxa and performed a numerical and cladistic analysis of variation using morphological (and some chemical) characteristics (Binns et al., 2002a). Natural populations were tentatively identified in the field according to McGregor's taxonomy (1968). Voucher specimens were deposited at the Department of Agriculture Ontario Herbarium (Ottawa, Canada) and experimental plants and seed were grown in a greenhouse (Binns et al., 2002a, 2002b, 2002c). Measurements for 81 morphometric traits (binary, quantitative, semiquantitative, and qualitative) were entered in a matrix to determine the degree of relationship and clustering between specimens, without any *a priori* weighting according to previous taxonomic identification. The data for seven traits were omitted due to missing values, resulting in a data matrix of 321 individuals by 74 characters (traits). An index of overall similarity was calculated for each pair of individuals (Gower, 1971). Several clustering methods were used to determine close relationships and potential taxonomic clusters (see Binns et al., 2002a, for details). Clusters generated in these analyses represented potential taxonomic entities. We evaluated clusters using

canonical discriminant analysis (Kshirsagar, 1972) to determine the relative strength of different cluster solutions. We also took the limitations of each clustering method into consideration, such as bias of equal-sized clusters, equal variance for members of a cluster, and misrepresentation of relationship between clusters due to error associated with extreme linkage methods. Herbarium specimens were also analyzed without *a priori* identification, including many from McGregor's collection. Finally, we also tested McGregor's taxonomy by using his keys to identify and label each specimen in our matrix of similarities, and then applying clustering strategies and assessing the validity of these solutions.

The morphometric analyses supported two acceptable cluster solutions based on overall similarity between specimens. The first revealed strong statistical support for two major divergent taxa within *Echinacea*, which we determined to be at a subgenus level. The species known currently as *E. purpurea* (L.) Moench was the sole taxon in *Echinacea* subgenus *Echinacea*, which contains only the species *E. purpurea*, and all other infrageneric taxa were in *Echinacea* subgenus *Pallida*. The second most acceptable cluster solution supported four taxa, which we determined to be at the species level: *E. purpurea* [=*Echinacea purpurea* (L.) Moench *nom. cons. prop.*; see Binns et al., 2001b], *E. laevigata* [=*E. laevigata* (Boynton & Beadle) Blake], *E. atrorubens* and *E. pallida*. There was also significant statistical support for an eight-cluster solution based on *a priori* labeling of each specimen using McGregor's (1968) taxonomy. The eight groups correspond to varieties within two species. The varieties of *E. pallida* follow: (1) *E. pallida* var. *angustifolia*; (2) *E. pallida* (Nutt.) Nutt. var. *simulata* (McGregor) Binns, B.R. Baum & Arnason [=*E. simulata* McGregor]; (3) *E. pallida* (Nutt.) Nutt. var. *tennesseensis* (Beadle) Binns, B.R. Baum & Arnason [=*E. tennesseensis* (Beadle) Small]; (4) *E.* pallida (Nutt.) Nutt. var. *sanguinea* (Nutt.) Gandhi & Thomas [=*E. sanguinea* Nutt.]; and (5) the type variety, *E. pallida* var. *pallida*. The varieties of *E. atrorubens* include (1) *E. atrorubens* Nutt. var. *paradoxa* (Norton) Cronq. [=*E. paradoxa* (Norton) Britton var. *paradoxa*]; (2) *E. atrorubens* Nutt. var. *neglecta* (McGregor) Binns, B.R. Baum & Arnason [=*E. paradoxa* (Norton) Britton var. *neglecta* McGregor]; and (3) the type variety, *E. atrorubens* Nutt. var. *atrorubens* Cronq. [=*E. atrorubens* Nutt].

Echinacea subg. *Echinacea* has the following key characteristics that distinguish between the two subgenera: fibrous roots, basal leaves usually 50 to 100 mm wide (rarely to 150 mm) and cauline leaves usually 45 to 90 mm wide, leaf blade trichomes bicellular with basal cell cylindrical and distal cell acuminate, branched major leaf veins, and an involucre with four series of bracts. *Echinacea* subg. *Echinacea* is distributed in sparse natural populations throughout the eastern and southeastern United States in a range that overlaps geographically, but rarely ecologically, with all species in *Echinacea* subg. *Pallida*. In Figure 1.1, a map of the distribution of each revised species, subg. *Echinacea* is shown as the range of *E. purpurea* (Binns et al., 2002a). For descriptions of each revised species and variety, as well as two morphological identification keys and other distribution maps, see Binns et al. (2002a).

The evolutionary relationships between the four species were estimated using cladistic analyses of 36 characters (including some phytochemical ones). In Figure 1.2, the monophyletic clade *Echinacea* is distinct from the outgroup *Rudbeckia* (98% bootstrap value). Within the *Echinacea* clade, the relationship was closest between *E. atrorubens* and *E. pallida*, which share three unique, derived characteristics, and *E. purpurea* was most basally divergent. The taxon *E. laevigata* was more closely related to *E. pallida* and *E. atrorubens*, based on the current data, although historically it was confused with *E. purpurea*.

In summary, we proposed a hierarchy of two subgenera, four species, and six varieties in the genus *Echinacea* from our morphometric numerical analyses (Binns et al., 2002a). The two subgenera are novel, but the four species groups were first suggested based on intuitive taxonomic methodology (Cronquist, 1945), and taxa at the variety level were all previously either species or varieties according to McGregor (1968). The revised taxonomy recognizes all of McGregor's taxa, except for one variety, *E. angustifolia* DC. var. *strigosa* McGregor. This putative variety may be a morphotype established from introgressed hybrids with similar phenotypic development in similar ecological zones, but not found to be distinct from *E. pallida* var. *angustifolia* (Binns, 2001). The

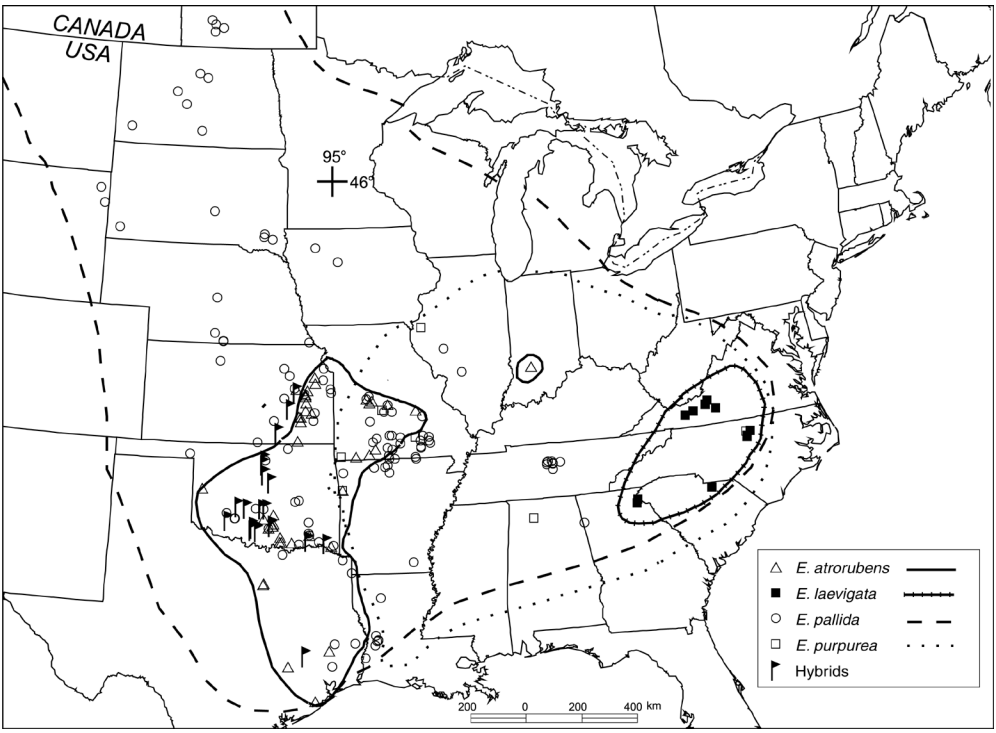

FIGURE 1.1 Distribution of four revised *Echinacea* species (including their varieties) from a combination of modern collections and herbarium specimens. Contour lines indicate predicted occurrence based on historical data as well as current surveys at the time of publication. (From Binns, S.E. et al., *Syst. Bot.*, 27(3): 610–632, 2002a. With permission.)

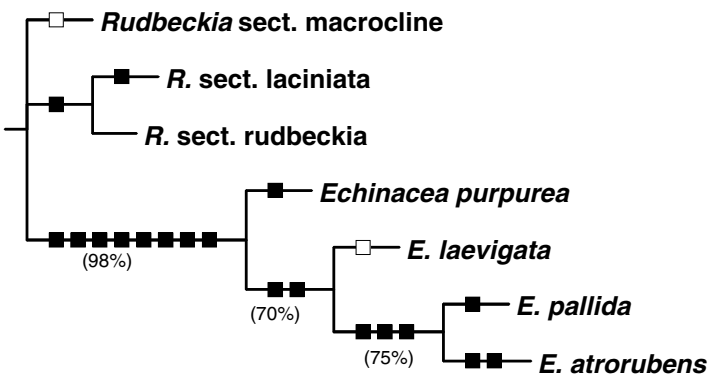

FIGURE 1.2 A 40-step most parsimonious cladogram representing the monophyletic genus *Echinacea* Moench compared to three sections of *Rudbeckia* in an outgroup. Bootstrap values using the 50% majority-rule consensus method are indicated in brackets below the branches. Cladistic analysis was performed with 36 characters. Dark boxes signify synapomorphies and empty boxes signify parallelisms. (Modified version from Binns, S.E. et al. *Syst. Bot*., 27(3): 610–632, 2002a. With permission.)

classifications of both McGregor (1968) and Cronquist (1945, 1955, 1980) are compared to the revised taxonomy in Binns et al. (2002a).

OTHER RECENT TAXONOMIC STUDIES

There have been other recent taxonomic treatments of *Echinacea*. In one such taxonomic review, McKeown (1999) recognized the misrepresentations of infrageneric taxa in *Echinacea* in the literature and one misleading key trait in previous works (i.e., ligule color is not a reliable diagnostic character due to its plasticity). McKeown (1999) described and mapped current, accurate geographic ranges for natural populations of *Echinacea*, and identified them according to McGregor (1968). McKeown's extensive germplasm collections have contributed to the U.S. Department of Agriculture (USDA) National Plant Germplasm System, which serves as a genetic resource base for conservation and research, including our *Echinacea* numerical taxonomic study (Binns et al., 2002a).

Another recent taxonomic assessment was the chemotaxonomic classification of only three putative species from 10 populations using multivariate data analysis by Lienert et al. (1998). This study was not comprehensive enough to elucidate infrageneric taxonomic structure. Urbatsch and colleagues conducted two analyses of molecular variation in the tribe Heliantheae (which contains *Echinacea*) (Urbatsch et al., 2000; Urbatsch and Jansen, 1995). The 1995 study of cpDNA restriction site variation revealed little diversity within the genus *Echinacea*, and both studies used very small sample sizes and failed to include all putative taxa, such as *E. pallida* var. *angustifolia*. However, the cladistic analysis based on internal transcribed spacer-region sequence data (Urbatsch et al., 2000) was more resolved and supported statistically than the cpDNA study (Urbatsch and Jansen, 1995).

Kapteyn et al. (2002) analyzed molecular variance using random amplified polymorphic DNA (RAPD) technology to distinguish between the three commercial taxa, *E. purpurea, E. pallida* var. *angustifolia*, and *E. pallida* var. *pallida*. They used *E. atrorubens* var. *atrorubens* as an outgroup. Although the sample size was adequate, the accessions in this study were all weighted by *a priori* identification and tested for their degree of cohesiveness as distinct groupings, similar to applying McGregor's identification to our data set (Binns et al., 2002a). When the conclusions of the three most recent taxonomic treatments are compared (Binns et al., 2002; Kapteyn et al., 2002; Urbatsch et al., 2000), there is one significant commonality that is shared by all three: cladistic analyses revealed a close relationship between the taxa *E. pallida* var. *pallida* and *E. atrorubens* var. *atrorubens*, joined by a more distant branch into a cluster with *E. purpurea*.

GERMPLASM ENHANCEMENT AND APPLICATIONS OF THE REVISED TAXONOMY

Two *Echinacea* taxa have been placed under federal protection by state heritage programs, namely *E. pallida* var. *tennesseensis* and *E. laevigata*. Other taxa in the genus that have been noted as endemic in particular habitats are *E. atrorubens* var. *neglecta* and *E. atrorubens* var. *paradoxa*. Some states, such as Missouri, have legalized a moratorium on roadside collection of any *Echinacea* taxon, except by permission for purposes of research and education. The revised taxonomy (Binns et al., 2002a) includes useful keys that can be used to improve identification of wild and cultivated germplasm, especially important for protecting those rare endemics and endangered taxa. In turn, this will enable effective conservation of the natural variation in this important plant genus.

It is important for growers to recognize that taxonomic errors may have occurred in past lots of certified seed, as well as the sale of mixed wild and cultivated seed. While the revised taxonomy does not introduce newly defined taxa, it provides a more accurate means to assess wild seed sources, and it introduces new name combinations for varieties, some of which were previously known at the species level. Applying the revised taxonomic names and using the new keys promises to greatly improve botanical certification of *Echinacea* germplasm (including potential hybrid lines), which has long plagued *Echinacea* cultivation around the world.

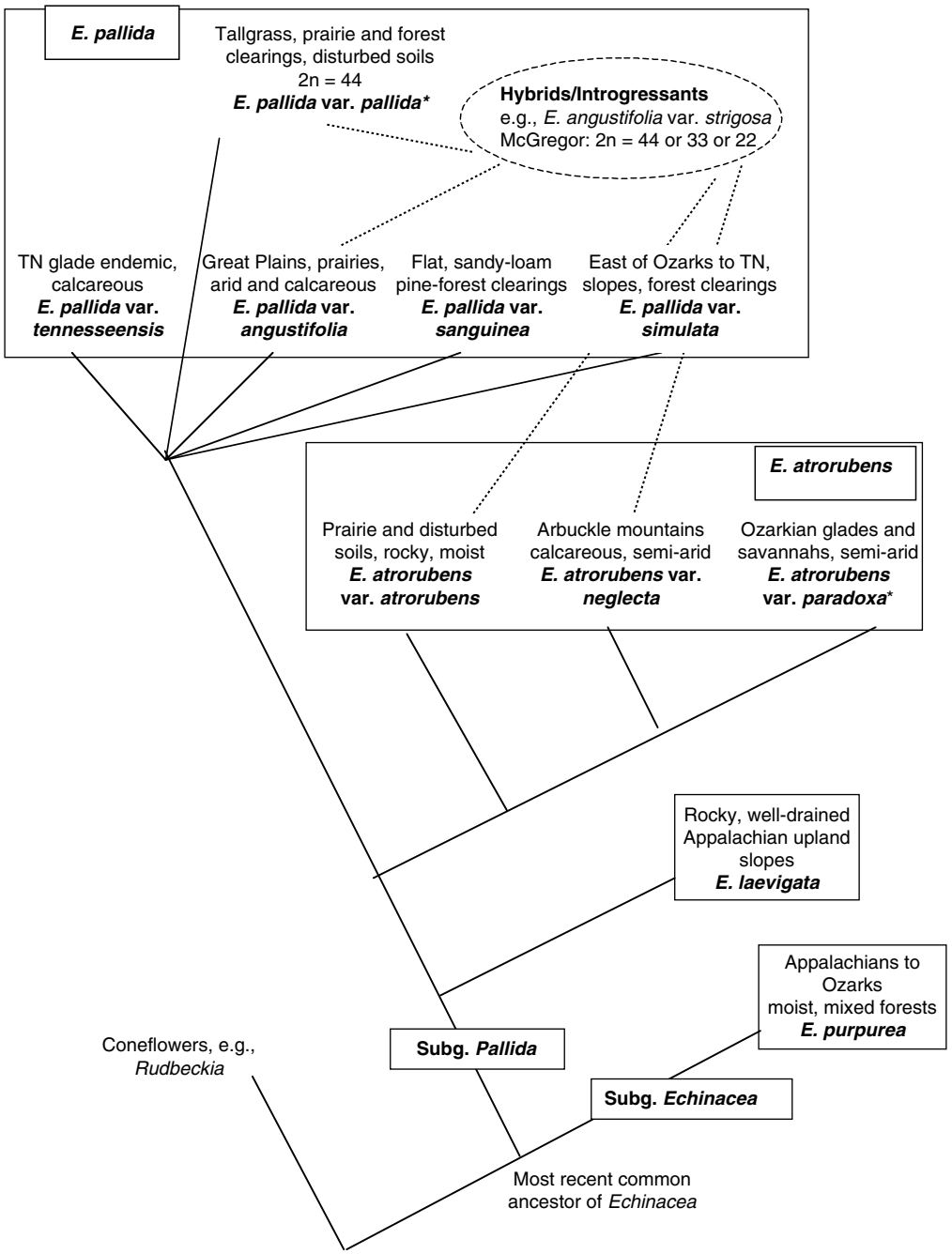

FIGURE 1.3 A hypothesized phylogeny of taxa within the genus *Echinacea* Moench based on a synthesis of current and historical geographical and ecological data. Branch nodes indicate putative ancestors, and branch lengths are not indicative of evolutionary distance. *Asterisk* (*) indicates sympatric populations; 2n is ploidy of regular somatic cells in plants (McGregor, 1968). (From Binns, S.E., 2001. The taxonomy, phytochemistry and biological activity of the genus *Echinacea* [Asteraceae], Ph.D. thesis, University of Ottawa. With permission.)

All organisms that are closely related, but not identical, contain variations that can be attributed to both genetic and environmental components. Genetic variation within and between certain taxa in the genus *Echinacea*, with corresponding phylogenetic implications, has been reported (Urbatsch et al., 2000; Baskauf et al., 1994; Kapteyn et al., 2002). Now, the tools and groundwork have been laid for genomic studies and determination of genetic markers necessary for breeding and elucidation of evolutionary relationships in the genus. At the time of our morphometric taxonomic revision, evidence was compiled from all phylogenetic and ecological literature on the subject to hypothesize the evolutionary basis for relationships among taxa in the genus *Echinacea* (Figure 1.3). From investigations by our group and other researchers (Baum et al., 2001), it was found that even *Echinacea* in cultivation for many years contained considerable phytochemical variation between individual plants. Safety and regulation of phytomedicines requires that phytochemical variation be reduced. Quality assurance can be partially addressed through germplasm enhancement to develop consistently characterized plant materials and to minimize phytochemical variation in crop situations. For instance, identification of specific genetic markers leading to phytochemical variation may enable optimal selection of genetic resources for cultivation. Also, the environmental variation imposed externally in the plant production process needs to be factored into improved cultivation methods. Botanicals used for medicine should not be treated with the same methods for safety and quality control that are used to regulate synthetic drugs.

The new, functional morphological taxonomy is invaluable in the germplasm enhancement process, not only for growers and botanists, but also for phytochemists and molecular biologists who rely on botanically characterized source materials. Ultimately, germplasm enhancement through multidisciplinary efforts offers the capacity to effectuate large-scale propagation of well-characterized, elite *Echinacea* cultivars, and thus to improve consistency and reproducibility in clinical trials with *Echinacea* phytomedicines.

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2 A Review of Preliminary *Echinacea* Genetics and the Future Potential of Genomics

Kathleen A. McKeown

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INTRODUCTION

In a survey of over 200 articles on *Echinacea* taken from the mainstream scientific literature between January 1998 and June 2002, only 3% of the publications reported specifically on genetic research. The majority of citations (55%) focused on human therapeutic or pharmacological investigations, 20% were of agricultural or horticultural interest, 18% were phytochemical analyses, and the rest were miscellaneous. Considering the history of use of this genus and its potential economic value, the paucity of genetic studies is striking. The literature emphasis may certainly be justified by the recent need to investigate the scientific basis of human pharmaceutical applications for *Echinacea*, and the corollary need to cultivate the relevant species and to characterize their biochemistry. However, we are working in an era of rapid gene discovery, of structural, functional, and biochemical genomics, and the dearth of information on even basic genetics should be a concern to all in the *Echinacea* research community. It would be timely and pragmatic to begin characterizing the genetic basis of *Echinacea* phytochemistry, to identify the structural (biosynthetic enzyme encoding) and regulatory genes, the proteins and pathways generating its natural products, and to ultimately understand the molecular, developmental, and environmental signals controlling the expression and variability of its complex suite of biochemical traits. Competitive advances in pharmaceutical and other economic uses of *Echinacea* will hinge on genetic and genomic characterization of this genus.

U.S. *ECHINACEA* GERMPLASM COLLECTION

My introduction to the genus *Echinacea* came as the result of collecting 88 of the 150 accessions now maintained by the U.S. Department of Agriculture's (USDA) Agricultural Research Service for the National Plant Germplasm System of the United States. This germplasm represents a comprehensive sampling of *Echinacea* diversity and includes all species recognized by McGregor

(1968) in his early taxonomic monograph. As of this writing, the wild seed of 80 *Echinacea* accessions has been increased in controlled pollination cages and is now available through the Germplasm Resources Information Network (GRIN) at http://www.ars-grin.gov/npgs. During the germplasm cultivation in a common field over several years, morphological characters were evaluated and taxonomic identification per McGregor (1968) was verified; this information is accessible through the GRIN database (Widrlechner and McKeown, 2002). Further evaluation of the germplasm is underway, including a root phytochemical analysis of these same 80 accessions in the laboratory of James Simon at Rutgers University.

During the germplasm collection, morphological types that had not been fully characterized from geographic areas of probable hybridization were observed (McGregor, 1968; McKeown, 1999). It is a common misconception that plant hybrids simply display intermediate morphological characteristics (Rieseberg and Carney, 1998). In fact, hybrids reflect varying degrees of parental, intermediate, transgressive, and novel traits, a phenomenon that can compromise the utility of morphological traits in taxonomic identification (Rieseberg and Carney, 1998). However, hybrids do tend to be complementary (additive) in expression of chemical characters, and can also display incomplete complementation, loss of parental compounds, and gain of novel chemistry (Rieseberg and Carney, 1998). Differences in the genetic basis of these two types of traits, quantitative for many morphological characters and single gene for many chemical compounds, can help explain these phenomena (Rieseberg and Ellstrand, 1993). Several USDA accessions now categorized as hybrids in the GRIN database exemplified this type of phenotypic complexity (Widrlechner, 2001). The observation of native hybrids and the documentation of polyploidy (McGregor, 1968) suggest the interesting possibility of hybridization as a speciation process in the genus. Current and evolutionary gene flow among populations and the possible hybrid origin of *Echinacea* species may become prominent factors in conservation, analyses of diversity, systematics, phylogenetics, and other areas of research, including phytochemistry.

Given the above observations, a few comments should be made on *Echinacea* systematics. A revision of the McGregor taxonomy (Binns et al., 2002) notwithstanding, I have found McGregor's 1968 classification to clearly distinguish *Echinacea* species in the greenhouse, phytotron, and field with the exception of the aforementioned natural hybrids. McGregor, who spent 15 years collecting data directly from wild stands of *Echinacea*, many of which have disappeared (McGregor, 1997), worked in the days prior to the development of phenetics and cladistics and prior to the extensive digging that now characterizes the fragmented and attenuated American prairie. His vast and valuable observations are literally unrepeatable due to wild population loss and decline over the last 35 years. It is unfortunate that taxonomic misunderstanding has arisen (Binns et al., 2002), but this is not attributable to McGregor. Given the power of molecular systematics, the true issue here is whether a morphometric study such as that of Binns et al. (2002) is the appropriate approach for a modern revision of *Echinacea* classification. The possibility of hybridization as a process in *Echinacea* speciation and the existence of polyploids will require a molecular systematic investigation of both chloroplast and nuclear genomes to elucidate phylogenetic relationships within the genus. Such a study is now under way in the laboratory of Jonathan Wendel and colleagues at Iowa State University in Ames, Iowa. The morphometric analysis of Binns et al. (2002) will certainly be of value in evaluating concordance between morphological and molecular characters; however, a fully revised systematic classification should also include molecular data. For the time being, therefore, I continue to follow McGregor's taxonomic treatment and will do so in this text.

PRELIMINARY MEASURES OF GERMPLASM DIVERSITY

Two types of multilocus markers—random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995)—have been utilized in preliminary assessments of *Echinacea* diversity. Wolf et al. (1999) developed RAPD markers specific for the commercial species *E. purpurea*, *E. angustifolia*, and *E. pallida* and demonstrated

the utility of these markers in distinguishing root mixtures of the latter two species. Kapteyn et al. (2002) also developed reproducible and diagnostic RAPD markers for the same species and for *E. atrorubens*, and further extended their analysis to an evaluation of genetic diversity. Similar levels of overall diversity for each of the commercial species were detected (Kapteyn et al., 2002). Notable was the analysis of molecular variance (AMOVA) result that 78.2%, 82.6%, and 98% of genetic variation occurred within *E. angustifolia*, *E. pallida*, and *E. purpurea* populations, respectively, rather than among them (Kapteyn et al., 2002). The high 98% within-population variance component signified no differences among the sampled *E. purpurea* accessions, a confounding result given their different commercial sources and history of selective breeding (Kapteyn et al., 2002). However, there were significant differences among some populations of the other two species, more so for *E. angustifolia* than for *E. pallida*, suggesting that certain accessions may be valuable breeding resources (Kapteyn et al., 2002). Also note that with only four individuals analyzed per accession (Kapteyn et al., 2002), differences among accessions may not have been fully resolved with the RAPD markers (Lynch and Milligan, 1994); see additional comments below.

Baum et al. (1999) were the first to utilize the AFLP technique (Vos et al., 1995) to assess diversity in *Echinacea*. Based on a preliminary study, they concluded that the cultivated *E. purpurea* of Trout Lake Farm, Trout Lake, Washington, had greater diversity than that of the wild species examined, which included *E. angustifolia*, *E. pallida*, *E. paradoxa* var. *paradoxa*, *E. sanguinea*, and *E. simulata* (Baum et al., 1999). This is unusual in that wild relatives are normally the reservoirs of greater genetic diversity (Chapman, 1989; Harlan, 1984). Clear interpretation of these data is hampered by the absence of wild *E. purpurea* in this study. Although the data show that commercial *E. purpurea* had the highest number of polymorphic sites, this number is dependent on sample size, which ranged from 2 individuals for *E. paradoxa* var. *paradoxa* to 55 for *E. purpurea*. Moreover, an intermediate (not maximum among the sampled species) value of average gene diversity over loci was calculated for *E. purpurea* (Baum et al., 1999); this is a more typical comparative measure. AFLP markers are particularly powerful (Mueller and Wolfenbarger, 1999) and the results of Baum et al. (1999) are certainly evidence of genetic diversity in *Echinacea*; however, more data are needed for comparisons among the species with these markers.

The expanded use of molecular markers in characterizing *Echinacea* will have great utility in diversity surveys; population, conservation, and evolutionary genetics; fingerprinting; genetic mapping; hybrid identification; and systematics (Karp et al., 1996; Milligan et al., 1994; Rieseberg and Ellstrand, 1993; Mueller and Wolfenbarger, 1999). All molecular markers have limitations, however, and appropriate applications, interpretations, and statistical analyses must be considered (Karp et al., 1996; Mueller and Wolfenbarger, 1999). This is particularly relevant to dominant markers such as RAPDs and AFLPs; given limited sample sizes, the above studies are preliminary and statistics can be biased even with data-pruning corrections (Lynch and Milligan, 1994). Additionally, levels of genetic variation are known to vary by marker (Russell et al., 1997), making the genetic diversity of *Echinacea* best assessed with multiple markers and techniques (Fritsch and Rieseberg, 1996).

POPULATION GENETICS

Although the study of plant population genetics may have no direct bearing on the economic development of *Echinacea*, it is a field directly applied to the analysis of genetic variation and to the conservation of both *in situ* and *ex situ* populations. Population genetic analyses elucidate patterns of genetic variation as it is distributed within and among populations of a species (Hamrick and Godt, 1996). These analyses rely on clear measures of genotypic and allelic diversity, traditionally estimated from the frequencies of co-dominant allozyme markers. Population structure, the departure from expected Hardy–Weinberg equilibrium within a population, is usually characterized by the well-known F-statistics developed by Wright (1951). (See Hartl and Clark (1997) for a detailed background.)

Allozyme data yielding estimates of genotypic frequency and allelic diversity have been collected for both of the federally endangered species, *E. tennesseensis* (Baskauf et al., 1994) and *E. laevigata* (Apsit and Dixon, 2001). Baskauf et al. (1994) compared population structure and diversity estimates of the endemic *E. tennesseensis* with that of its widespread congener, *E. angustifolia*. This comparative method was adopted in order to avoid the confounding effects of phylogenetic differences between species from different genera (Baskauf et al., 1994; Gitzendanner and Soltis, 2000). *E. tennesseensis* had significantly less genetic variability than did *E. angustifolia* (Baskauf et al., 1994). F-statistics indicated that less than 10% of the total genetic variation for each species was due to differences among populations (Baskauf et al., 1994). This is reasonably consistent with the findings of Kapteyn et al. (2002) and corresponds to the strong genetic similarity among populations for a given species per calculations of genetic identity (Baskauf et al., 1994).

Echinacea laevigata is the other rare and endangered taxon in the genus, with 24 known, extant populations endemic to the southeastern United States (Apsit and Dixon, 2001). Measures of both genotypic and allelic diversity indicated that the 11 populations sampled are well differentiated and that conservation of all populations would be the ideal approach to preserving the total range of genetic diversity of this species (Apsit and Dixon, 2001). Note that the "among population" component of variance in this study was 78%, indicating that most of the genetic variability was caused by differences between populations (Apsit and Dixon, 2001). This is dramatically different from the results of Baskauf et al. (1994) and Kapteyn et al. (2002) for other species. Many factors can contribute to population differentiation, including genetic drift resulting from reduced population size and limited gene flow from isolation. See Apsit and Dixon (2001) for an excellent discussion of such factors.

Wagenius (2000) studied the diversity and "fine-scale" population substructure (by mapping individual plants within subpopulations) of wild *E. angustifolia* in a fragmented prairie region of western Minnesota. He found a strong positive linear correlation of several important estimates of genetic diversity (proportion of polymorphism, allelic richness, and gene diversity) with population size, that is, the smaller the population, the less diversity (Wagenius, 2000). Small local populations also had more spatial genetic structure, indicating a reduction in random mating (Wagenius, 2000). Wagenius (2000) concluded that small population size was a factor in lowered fitness as measured by pollen limitation (the absence of compatible pollen inferred from style persistence) and progeny vigor. It would be interesting to see how values of genetic identity and interpopulation distances as measured by other molecular markers compared to these results.

A few comments on mating systems in *Echinacea* are warranted here. *E. angustifolia* has been shown to have the potential for a mixed mating system including xenogamy (out-crossing between individuals), geitonogamy (self-fertilization between florets of the same individual), and autogamy (self-fertilization within a floret) in a native prairie of southwestern South Dakota (Leuszler et al., 1996). McGregor observed a small degree of self-mating in all *Echinacea* species (McGregor, 1997). The assumption of a self-incompatibility system (not yet genetically characterized) in *Echinacea* as in other Asteraceae is reasonable; however, given that incompatibility systems can be leaky, as demonstrated in other composites (Cheptou et al., 2002; Young et al., 2000) and by *E. angustifolia*, a mixed mating system in *Echinacea* would not be surprising. Also, reduced population size in out-crossing species can lead to increased self-crossing (Reinhartz and Les, 1994). To add to this complexity, *E. laevigata* reproduces clonally (Apsit and Dixon, 2001; Edwards, 1997), as do other species in cultivation (McKeown, unpublished observation). A detailed study of *Echinacea* mating systems will be essential to a full understanding of its native diversity.

PHYLOGENETICS

Phylogenetics, the study of the evolutionary history of a group of organisms, provides another perspective on genetic diversity, one that I think is particularly relevant to *Echinacea*. Given the degree of morphological similarity between the species, the relatively young evolutionary age of

this genus—as compared to its "cousin" genus *Helianthus* at 30 to 60 million years (Rieseberg, 2001)—the hybridization between extant populations and its possible role in speciation, what information can be gleaned from the study of molecular variation in an evolutionary context?

A primary goal of molecular systematics is the accurate reconstruction of phylogenetic relationships. Several systematic studies of the coneflower genera have included a limited sample of *Echinacea* species. Both chloroplast restriction site variation (Urbatsch and Jansen, 1995) and sequence divergence of the nuclear ITS-1, ITS-2, and intervening 5.8S regions (Urbatsch et al., 2000) have revealed relatively low divergence among a number of *Echinacea* species. These studies were not intended to fully explore *Echinacea* molecular systematics, but give clues to the challenges ahead. Low molecular variation is the bane of the molecular systematist, but low divergence at the molecular level can also indicate recent divergence of taxa given the appropriate analytical context.

Phylogeography utilizes the genealogy of alleles and the geographic patterns of their lineages to identify evolutionary processes underlying the distribution of species (Avise, 2000). It is an analytical tool that has been widely utilized in animal systems and is gaining ground in plant evolutionary and biogeographic studies (Schaal et al., 1998). The power of phylogeography lies in its link to both micro- and macroevolutionary disciplines; common ancestry, patterns of divergence and genetic exchange, when mapped on contemporary distributions, can illuminate the biogeography of species (Avise, 2000). I have initiated a broad phylogeographic study of *Echinacea* using nucleotide sequence variation in the chloroplast genome. Preliminary data indicate a shallow divergence among the taxa, consistent with the chloroplast restriction site data of Urbatsch and Jansen (1995), and a broad bipartite geographical partitioning of gene lineages on either side of the Ozark Mountains that is independent of species identity. The pattern is suggestive of a rapid range expansion (Avise, 2000) that might be consistent with the spread of prairie forbs during the Hypsithermal Interval of the Holocene (Baskin et al., 1997). I must emphasize that these are preliminary data; the full analysis will be published elsewhere. Additionally, the Ozarks have been earmarked as the center of diversity and possible origin of *Echinacea* as based on morphological variation (Baskin et al., 1997; Binns et al., 2002; McGregor, 1997; McKeown, 1999); the phylogeographic data have potential to support this common observation. Upon completion, the genus-level phylogeography will hopefully contribute to the broad evolutionary story of *Echinacea* in North America.

THE POTENTIAL OF GENOMICS

Consider the multiple chemical isomers of a given class of compounds, such as the extensively characterized alkamides (Bauer and Foster, 1991; Bauer and Remiger, 1989; Bauer and Wagner, 1991; Bauer et al., 1988a, 1988b, 1989, 1990; Binns et al., 2002; Dietz and Bauer, 2001). How many genes are involved in this phytochemical expression? The effects of environment aside, is the array of different alkamides the phenotypic result of the action of many, possibly related genes encoding many enzymes? Or does this chemotypic variation result from fewer genes encoding fewer enzymes that are not substrate specific, but flexible enough in substrate binding and the active site mechanism to yield the broad phenotypic array of compounds found within this chemical class? Secondary metabolic enzyme multiplicity and flexibility are not uncommon. For example, different isomers of chalcone isomerase from *Glycyrrhiza echinata* act on different substrates (Kimura et al., 2001), and several terpene synthases synthesize multiple products from a single substrate (Bohlman et al., 1998). Orthologs of these enzymes may certainly be found in *Echinacea*, but we have yet to identify a single biosynthetic enzyme responsible for its valuable phytochemistry.

Consider now a broader view of the *Echinacea* genome. Here is a set of genes and regulators under transcriptional control that produces an array of enzymes and a network of biochemical pathways, which themselves produce a flux and array of intermediates and end-product phytochemicals, operating within an intrinsic system of interactions and developmental cues at all levels, which is modifiable by the extrinsic environment. No biological system is understood in this

daunting detail. The hard work of teasing apart the economically relevant pathways lies ahead for *Echinacea*. Yet secondary metabolic components of this genome are within reach. Most notably, progress is being made on the regulation and pathways of phenylpropanoid metabolism, the source of plant phenolics (Weisshaar and Jenkins, 1998), and genes involved in the biosynthesis of unsaturated fatty acids, suggested as the precursors of plant alkamides (Greger, 1984), have been identified from an *Arabidopsis* EST (expressed sequence tag) database (White et al., 2000). The point here is that published sequences of biosynthetic enzymes from other plants are doorways, through sequence similarity, to those of *Echinacea*. Coming down the pipeline are EST databases of *Helianthus* and *Echinacea* (Knapp, 2003) as part of the Compositae Genomics Project (http://compgenomics.ucdavis.edu). Synteny (gene order and organization) is another doorway into the genome that can enable the isolation and characterization of genes (Somerville and Somerville, 1999). Metabolic profiling (metabolomics) conducted in parallel with microarray analysis and proteomics has enormous potential for elucidating the circuitry of gene-protein secondary product pathways, feedback mechanisms, and genetic controls (Trethewey et al., 1999).

Finally, there is the impact of environment and development on secondary chemistry that has been previously foregone in this discussion. What are the factors that influence the genetic potential of *Echinacea* to produce its phenotypic range of phytochemical characters? And what are the true limits of that range, in terms of classes of compounds shared by species of chemical isomers within classes and their quantities? Why are these questions important? It has to do with our ability to maximize applications of *Echinacea* phytochemistry. Microarray analysis and metabolomics may answer these questions through identification of active genes and secondary metabolites associated with plant developmental stages and as influenced by environmental treatments and controls (Somerville and Somerville, 1999). Moreover, given that there may not be a singly active pharmacological metabolite, but a complex of (synergistic?) bioactive compounds, metabolomics may be the best approach to optimizing pharmaceutical products.

Echinacea phytochemistry is an economically important and complex set of phenotypic traits, the genetic architecture and control of which we know nothing. Underscore this with another point not heretofore mentioned, that there is not yet published a genetic (linkage) map based on recombination frequencies of markers or a physical map based on nucleotide sequence data. The linkage group (chromosomal) foundation and marker scaffolding on which genomics will rest—for a full genetic picture of this genus—still need to be constructed for *Echinacea*.

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Section II

Cultivation of Genus Echinacea

3 *In Vitro* Culture of *Echinacea* Species

Pamela S. Coker and N. Dwight Camper

CONTENTS

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INTRODUCTION

Plant tissue culture is the process of producing callus tissue, plant organs, or intact plants using a small piece of a donor plant or even a single cell. This process consists of using an artificial medium and exogenous growth hormones. Successful plant tissue culture is based on the cell theory of Schleiden (1838) and Schwann (1839), and the concept of totipotency (a plant cell is capable of regenerating a whole plant) (Gautheret, 1983). The portion of plant material used is referred to as the "explant." The explant may be from any portion of a plant (stem, leaf, nodal segment, etc.). Because this method is essentially a closed system, one may manipulate conditions for specific morphological attributes as well as for an increased yield of a particular chemical constituent (Figure 3.1). Mass production of clonal plants from a single highly desired plant is often accomplished through *in vitro* culture. *In vitro* culture may be used to obtain virus-free plant material by excising only the meristematic dome of a contaminated plant and using it as an explant source. *In vitro* systems may be used as a model to study various metabolic functions. In addition, transformation experiments are often accomplished in *in vitro* systems. For example, when products are known to be produced in roots, as is the case with *Echinacea* spp., hairy root cultures may be initiated using *Agrobacterium rhizogenes* as the vector.

Advantages of an *in vitro* system for medicinal plants include: (1) year-round availability of plant materials for extraction of pharmaceuticals produced under controlled conditions; (2) potential regulation of metabolic pathways from which active ingredients or marker compounds are derived; (3) potential genetic modification of cells/tissues to produce specific intermediates or metabolites (Kurz and Constabel, 1979); and (4) mass micropropagation of desired plants. Early attempts to produce secondary metabolites were reported in 1960; however, yields were generally low (Tulecke and Nickell, 1959). At times, the secondary product of interest is sequestered in a specific organelle and may not be produced in *in vitro* cultures. Recent information about signaling mechanisms in plants and the potential control of metabolite production — that is, polysaccharide elicitor from *Pseudomonas* sp. induced higher levels of rosmarinic acid in oregano shoots callus (Perry and Kalidas, 1999), elicitor induction of sesequiterpene production in tobacco, and *Hyocayamus muticus* cell culture (Back and Chappell, 1995) — poses potentially interesting possibilities for manipulation of cell cultures for the production of pharmaceutical compounds.

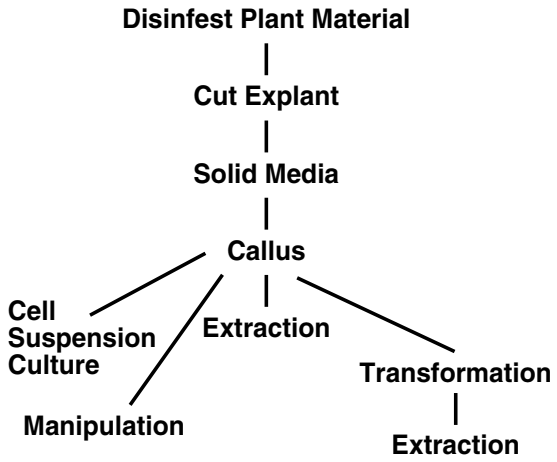

FIGURE 3.1 Schematic of general tissue culture protocol with emphasis on callus induction for cell suspension culture, extraction, or transformation/manipulation.

REGENERATION OF *ECHINACEA PURPUREA*

Establishing protocols for micropropagation and callus production of *Echinacea purpurea* L. Moench (purple coneflower) may be a way to recover some of the endangered and overharvested species of *Echinacea*, as well as a method for providing plant materials for extraction of medicinally important compounds. Regeneration of *E. purpurea* has been obtained from different explants. Choffe et al. (2000a) developed a method for inducing root organogenesis from hypocotyl and cotyledon explants of *E. purpurea*. Explant material was obtained from 14-day-old seedlings obtained from seeds that had been extensively disinfected prior to germination. Indole acetic acid (IAA) (5 and 15 to 20 µM) and indole butyric acid (IBA) (2.5 to 20 µM) were effective in inducing root formation from either hypocotyl or cotyledon explants. IBA was found to be the most effective auxin tested. The efficiency of auxins for root induction was IBA>IAA>NAA (naphthylene acetic acid). Choffe et al. (2000b) obtained regeneration from petiole explants from 2-month-old sterile seedlings cultured on medium with benzylaminopurine (BA) or thidiazuron (TDZ) in combination with IAA. Regeneration was observed either by direct somatic embryo formation on the epidermis of the petiole, or *de novo* from callus tissues formed in the subepidermal cell layers. Optimal BA level for regeneration from petiole explants was 2.5 µmol/L. When somatic embryos and shoots were separated from the explant tissue and subcultured on basal medium, more than 90% of all regenerates developed into intact plants.

Coker and Camper (2000) obtained callus, shoot, and root formation from hypocotyl explants on media with different hormone combinations. Disinfested *E. purpurea* achenes were placed in sterile distilled water for 1 to 2 hours to soften the achene prior to inoculation onto potato dextrose agar. Seedlings were harvested at 11 days and hypocotyl sections (4 to 5 mm) served as the explant source. The general medium consisted of Murashige and Skoog Minimal Organics (MSMO) (Sigma Chemicals, St. Louis, MO; Murashige and Skoog, 1962), 30 g/L sucrose, and 8 g/L agar at pH 5.7. Hormone regime consisted of 1 to 3 mg/L NAA plus 1 to 2 mg/L kinetin, or 0.5 to 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) plus 1.5 to 2.0 mg/L kinetin with subsequent transfer to hormone-free media. All samples were observed for callus, anthocyanin, shoot, and root production. Morphological development of hypocotyl explants varied with hormonal type and concentration. Anthocyanin production was monitored as a visual indicator that secondary metabolism was taking place, but no difference was observed between treatments. Generally, 2,4-D/kinetin combinations produced more callus than explants treated with NAA/kinetin combinations (Figure 3.2). The

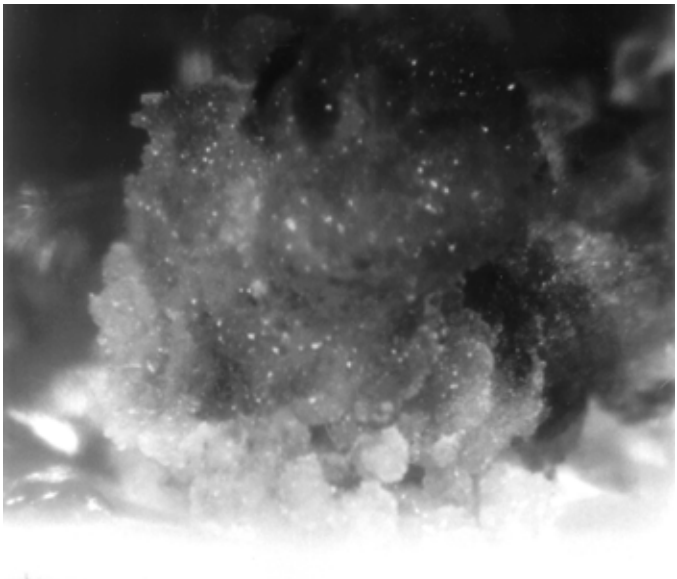

FIGURE 3.2 Callus obtained on media with 1.0 mg/L 2,4-D + 2.0 mgL kinetin after 45 days.

percentage of explants forming shoots and roots was higher on media with NAA/kinetin than on media with 2,4-D/kinetin. All combinations of 2,4-D/kinetin treatments induced more callus formation than NAA/kinetin combinations. The greatest number of plantlets was produced with 1 mg/L NAA and 1 mg/L kinetin (Figure 3.3). Regenerated plants produced flowers similar in color and shape to those of donor plants (Figure 3.4).

Harbage (2001) established a micropropagation method for *E. purpurea*, *E. angustifolia*, and *E. pallida*. Removal of seed coverings enabled production of contaminant-free cultures of *E. purpurea* and *E. angustifolia*, but not *E. pallida*. Shoot-tip explants were contaminated in all cases. Generally, shoot formation increased with BA concentration (0.45 to 4.45 μM) for all three species. Rooting was affected by species but not by light, temperature, or IBA concentration. Rooting could be induced in BA-free medium without auxin addition.

LIQUID CELL CULTURE

Liquid cell cultures can be derived from callus or directly from explant material. Establishment usually involves placing an explant on solidified nutrient medium supplemented with growth hormones for initial callus production. Explants form callus that is subsequently subcultured to increase mass and then transferred to liquid nutrient medium. Alternately, explant tissue (leaf, root, stem) can be placed in liquid nutrient medium to obtain a cell suspension. Liquid cell cultures provide a source of material for various metabolic studies or for transformation studies. Growth hormones in the medium can influence secondary product formation; for example, 2,4-D addition to the medium may accelerate callus formation, suppress subsequent morphogenesis, and prevent secondary product formation (Kurz and Constabel, 1979). Lower 2,4-D concentrations in combination with other hormones often favor secondary product formation (Gamborg et al., 1976).

Protocols were established in the late 1980s for liquid cell cultures of *E. purpurea* (Luettig et al., 1989; Wagner et al., 1988). Luettig et al. (1989) used the supernatants from liquid cell culture of *E. purpurea* to obtain highly purified arabinogalactans. The arabinogalactans were then used to measure macrophage activation in a mice assay. Wagner et al. (1988) studied immunologically active polysaccharides of *E. purpurea* from cell cultures. Leaf and stem explants were cultured in

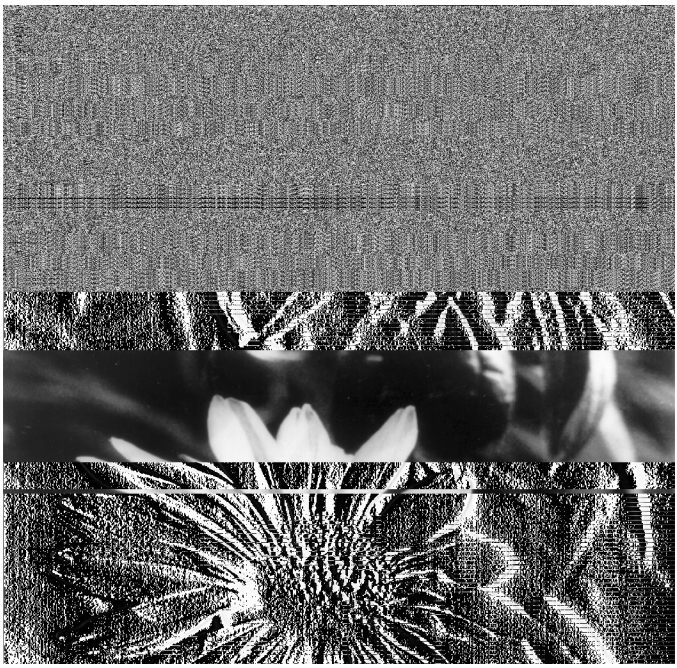

FIGURE 3.3 Shoot production on Murashige and Skoog Minimal Organics media with 1 mg/L naphthylene acetic acid plus 1 mg/L kinetin after 29 days.

FIGURE 3.4 Mature *Echinacea purpurea* tissue from *in vitro* cultured hypocotyl explants. (From *Herbs, Spices, Med. Plants*, Haworth Press, Binghamton, NY, 7(4), 2000. With permission.)

Linsmaier/Skoog medium with 2,4-D. Polysaccharides were extracted after a 14- to 21-day period and were then used in immunological assays. Three polysaccharides, two neutral furogalactoxyglucans, and an acidic arabinogalactan were detected and shown to be immunologically active. Proksch and Wagner (1987) concluded that the polysaccharides obtained with liquid cell cultures were structurally different from those found in intact *E. purpurea* plants. Similarly, Schollhorn et al. (1993) used cell suspension studies to provide polysaccharides for immunochemical investigations. In this study, a polyclonal IgG-antibody produced from rabbits was used to study the relationship between the polysaccharide structure and binding. Schollhorn et al. (1993) determined a high degree of structural similarity between the acidic arabinorhamnogalactan from the plant

material and the acidic arabinorhamnogalactan from the cell suspension. Like Proksch and Wagner (1987), Schollhorn et al. (1993) determined that the acidic heteroxylan and fucogalactoxyloglucan were structurally different between the plants and the cell suspension cultures.

Hairy root cultures can be induced in tissue cultures using inoculation with strains of *Agrobacterium rhizogenes*. Hairy root cultures can provide a source for the standardized production of secondary metabolites in several plant species (Signs and Flores, 1990). Trypsteen et al. (1991) transformed *E. purpurea* with several strains of *A. rhizogenes*. Two strains produced callus while the other two strains resulted in formation of hairy roots. Callus and hairy roots produced on the plants were analyzed as a possible source of isobutylamides (Trypsteen et al., 1991). Opine detection confirmed successful transformation. High performance liquid chromatography (HPLC) alkamide patterns for control and transformed tissues indicated the following: similar levels in control and transformed callus to that in root tissue and some differences in selected peak intensities; levels in transformed and control root tissue were similar with slight differences in selected peak intensities.

SUMMARY

Echinacea species have been successfully regenerated from several explants, hypocotyl (Coker and Camper, 2000; Choffe et al., 2000a), cotyledon, petiole (Choffe et al., 2000a), and shoot-tips (Harbage, 2001). Explants successfully used for liquid cell-suspension cultures include callus tissue, leaf, and stem (Wagner et al., 1988; Schollhorn et al., 1993). Root tissue has been successfully transformed by *Agrobacterium* to produce hairy root cultures and callus (Trypsteen et al., 1991). Potential uses for cultured material includes year-round plant availability, material for mass production of specific clonal lines or repopulation of endangered species, a source of virus-free plant material, as well as a source of material for transformation experiments. Cultured material may also be used as a model system for studying metabolic functions as well as to manipulate metabolic pathways and the production of metabolites. This cultured material may be used for mass production of secondary products (i.e., arabinorhamnogalactan [Schollhorn et al., 1993], heteroxylan, and fucogalactoxyloglucan [Proksch et al., 1987]).

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4 Cultivation in Europe

Bertalan Galambosi

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INTRODUCTION

Echinacea (Asteraceae), a North American genus of 11 recognized taxa, is of great contemporary economic and scientific interest. Three species—*Echinacea purpurea, Echinacea angustifolia*, *and*

Echinacea pallida—show potential pharmacological activity and have economical value all over the world (McKeown, 1999).

Echinacea is a relatively new genus in Europe. First, these species were introduced as decorative plants and later, from about 1930 to 1960, they became very popular as medicinal plants. As evidence of their medicinal value became clear, supplies derived from wild native American plants did not meet the increased demand. Thus, research efforts today are directed at establishing the best methods for cultivating *Echinacea* species in Europe and North America. More than 15 countries now have cultivation and production facilities.

From the several *Echinacea* species, the most studied and well known is the purple coneflower (*E. purpurea*), the species that has been most fully domesticated thus far. Several articles and books have been written on the biological activity, chemistry, and medicinal effects and uses of *Echinacea* (Bauer and Wagner, 1990; Foster, 1991; Hobbs, 1995), but the literature is sparse concerning cultivation and agrotechnical issues for the genus. Information on cultivation methods, yield components, and effects on biomass productivity and chemical components is very limited. A few papers dealing with agrotechnical issues were published recently in scientific and practical journals and in national herb cultivation handbooks.

The history of the cultivation of *Echinacea* in Europe can be divided into several periods. The first period started when *Echinacea* was first introduced into Europe as a garden perennial for its decorative qualities. John Banister introduced it into English gardens before 1699 (Ewan and Ewan, 1970). The earliest written report on *Echinacea* appeared in the 18th century, when the genus was described in the *Horticultural Lexicon* by Miller in 1776 as *Rudbeckia purpurea* (equivalent to *E. purpurea*). The first cultivation methods were described by Reichenbach in 1833. *Echinacea*-based drugs appeared in the European literature at the end of the 19th century. The first reports on medical utilization of *Echinacea* appeared in 1898 (*E. purpurea*) and in 1897 (*E. angustifolia*) (Bauer and Wagner, 1990).

In the second period, 1920 to 1940, several articles referring to *Echinacea* as a homeopathic herb were published in Germany (Schwabe, 1924). When the German companies Madaus and Schwabe started to use *Echinacea* as a medicinal plant on a larger scale, cultivation activity began on an equally large scale.

In the third period, 1950 to 1980, cultivation of *Echinacea* expanded to meet the increasing demand of medicinal manufacturers. Commercial cultivation began in Germany in Mittel-Unterfranken by Madaus and Schwabe, and in Inning and Weber-Ammersee by the Vogel company (Bauer and Wagner, 1990).

Facilities for cultivation were also established in Bocourt, Switzerland, by Spagyros, in Roggwill, Switzerland, by Vogel, and in Elburg, The Netherlands, by Biohorma Ag. Others were set up in northern Italy (Sudtirol-Gardasee) and in Yugoslavia and Spain (Boehringer-Ingelheim Company). A summary of all these cultivation methods and techniques on *Echinacea* can be found in Heeger (1956), and later by Ebert (1982).

Because of morphological similarities but vague taxonomical definitions, there was much confusion in the identification of species. In 1939, Madaus ordered seeds of *E. angustifolia* from America. The plants grown from those seeds later proved to be *E. purpurea*. This might be one of the main reasons for the intensive product development and research into *E. purpurea* in Europe.

The fourth period began in the mid-1980s when detailed taxonomical and agrotechnical research was carried out mainly in Germany. The first formal research on *E. purpurea* was done in Sweibheim (Barnickel, 1985). Later, long-term and basic agronomic research was carried out at Bayerische Landesanstalt fur Bodenkultur und Pflanzenbaues in Freising (Bomme, 1986; Bomme et al., 1996). Published results generated increasing interest in *Echinacea* in other European countries. Agronomic research and commercial cultivation extended into several European countries, such as Poland, Romania, Hungary, and Slovakia. Finally some Nordic countries also initiated research on *Echinacea*. The interest in *Echinacea* can be seen from the number of publications on *Echinacea* species in agriculture (Table 4.1). According to the available literature, 61 manuscripts were pub-

lished from 1951 to 2002. These statistics include only articles and monographs focused exclusively on *Echinacea*, and do not include handbooks of herb cultivation or general articles in which *Echinacea* is one among other herbs, such as in the paper by Bomme and Wurzinger (1990). In the 1990s, the number of publications increased, which paralleled equally intense research activities. Research activities in other countries increased as well during this period.

AGROTECHNICAL RESEARCH OF *ECHINACEA*

Germany

Research on *Echinacea* in Freising focused on the detailed cultivation technology of *E. purpurea* and *E. angustifolia*. The first basic production technology for these two species was published by Bomme in 1986. Fertilization studies were completed by Bomme-Wurzinger (1990), and Bomme-Nast (1998). Between 1986 and 1988, 10 *E. purpurea* cultivars were compared (Bomme et al., 1992a, 1992b). In 1999, one paper was published dealing with the seed treatments of three *Echinacea* species, and focused on direct drilling in the field (Gatterer et al., 1999).

Another research team led by Franke has been working on the technology for growing *E. pallida* since 1993 (Franke et al., 1997, 1999). *E. pallida* was also the object of the third research team in Thyringia, where the main agronomic elements have been studied (Fröbus et al., 1997). The principal questions of the agronomic studies in Germany are presented in Table 4.2.

Switzerland

To meet the demand for industrial raw material, agronomic research started in the first half of the 1980s. Seed biology and basic agronomic procedures were studied by Smith-Jochum and

TABLE 4.1
Agronomic Articles and Monographs on *Echinacea* Species Published in European Countries, 1951 to 2002

Country	1951–1960	1961–1985	1986–1990	1991–1995	1996–2000	2001–2002	Total
Germany	1	2	3	4	4		14
Italy			1		2	6	9
Slovenia			1		1		2
Switzerland			1	2			3
Finland				2	1		3
Russia			1	2	1		4
Romania				4			4
Bulgaria				1			1
Poland					10		10
Czech					1		1
Slovakia					3	1	4
Norway					1		1
Denmark					1		1
Lithuania					3		3
Great Britain					1		1
Total	1	2	7	15	29	7	61

Albrecht (1987, 1988). The studies focused on all three species. Optimization of the harvest in conjunction with the growth and plant phenology was studied by Heinzer et al. (1988). Identi-

TABLE 4.2
Publications on *Echinacea* Agrotechnology, Germany

Species	Subjects	References
E. pupurea	Agrobiology, cultivation methods	Heeger, 1956
		Bomme, 1986, 2000
		Bomme et al., 1992a, 1992b
	Germination, direct sowing	Gatterer et al., 1999
E. pallida	Short cultivation technology	Ebert, 1982
	Full cultivation technology	Bomme, 1986
	Germination, direct sowing	Gatterer et al., 1999
	Growing methods, cichoric acid	Franke et al., 1997, 1999
E. angustifolia	Growing methods, cichoric acid	Franke et al., 1997, 1999
	Cultivars	Schenk and Franke, 1996
	Germination, direct sowing	Gatterer et al., 1999
		Fröbus et al., 1997

fication of the three species, seed biology, and seed chemical profiles were reported by Schulthess et al. (1991).

Italy

Field experiments and cultivation started in northern Italy in the 1980s. The first results of fertilization on *E. angustifolia* were published by Tessari (1987), and on *E. pallida* by Bezzi and Tessari (1989). The commercial cultivation of *E. pallida* started on the ABOCA Herb Farm (7 ha) and resulted in the beginnings of the selection process (Fulceri et al., 2001). Germination problems, which occurred in practical cultivation, led to detailed investigations of *E. angustifolia* (Macchia et al., 2001). Recently, detailed agronomic studies have been carried out with three *Echinacea* species (Aiello et al., 2002a, 2002b, 2002c). The main issues studied in Italy are shown in Table 4.3.

Austria

Although no specific cultivation studies were carried out in Austria, *Echinacea* species have been cultivated in the Landes-Versuchsanlange für Spezialkulturen in Wies. The latest handbook of medicinal herb cultivation, including *Echinacea*, which summarizes research in Europe, was published by Austrian experts (Dachler and Pelzmann, 1994, 1999).

Slovenia

From 1970 to 1980, some agronomic research began in collaboration with Italian and German experts (Wagner, 1987). Rode (1996) studied the climatic suitability for *Echinacea* cultivation in Slovenia. Data on commercial cultivation areas are not available.

Romania

The first article on cultivating *Echinacea* was published in a herb cultivation handbook (Csedö, 1980). Systematic acclimatization studies were carried out in Romania by a research team led by Muntean at the University of Cluj-Napoca. Agrobiological and agrotechnical experiments were carried out on

TABLE 4.3
Publications on *Echinacea* Agrotechnology, Italy

Species	Subjects	References
E. purpurea	Virus diseases	Bellardi et al., 1997
	Yield, crop duration	Aiello et al., 2002a
	Transplantation times	Aiello et al., 2002b
	General importance	Aiello, 2002
E. angustifolia	Fertilization	Tessari, 1987
	Germination, seed dormancy	Maccia et al., 2001
	General importance	Aiello, 2002
	Yield, quality, crop duration	Aiello et al., 2002a
	Transplantation times	Aiello et al., 2002b
	Provenance comparison	Aiello et al., 2002c
E. pallida	Fertilization	Bezzi-Tessari, 1989
	Micropropagation, selection	Fulceri et al., 2001
	Yield, quality, crop duration	Aiello et al., 2002a
	Transplantation times	Aiello et al., 2002b

E. pallida and *E. purpurea* from 1988 through 1991 (Muntean et al., 1990, 1991, 1992, 1993). Recently, phytochemical investigations have been reported on *E. purpurea* (Radu et al., 2001).

POLAND

From 1990 through 2000, long-term and detailed experiments were carried out at the Medicinal Plant Research Institute, Poznan, and at several universities to improve the technology for growing *Echinacea* in Poland. Early research focused mainly on *E. purpurea*, and then more recently on *E. pallida* (Kordana and Mordalski, 2001).

On the basis of these experiments, a treatise on complex cultivation technology was published concerning its practical production (Mordalski et al., 1994). In Poland, *Echinacea* products have been produced for the domestic medicinal market and for export. Issues studied in Poland are shown in Table 4.4.

TABLE 4.4
Publications on *Echinacea* Agrotechnology, Poland

Species	Subjects	References
E. purpurea	Plant age, planting system, yield	Weglarz and Karaczun, 1996
	Harvest system, yield	Weglarz, 1998
	Flower biology	Seidler-L. and Dabrowska, 1996a
	Selection, breeding	Seidler-L. and Dabrowska,1996b
	Fertilization, fenolic acid content	Berbec et al., 1998
	Weed control	Kordana et al., 1996
	Direct sowing, transplanting, yield	Kordana et al., 1998
	Chemical weed control, plant protection, yield	Kucharski, 1997, 2000
	In vitro cultivation	Krajevska et al., 1996
	Full cultivation method	Mordalski et al., 1994
E. pallida	Growing methods, chemical compounds	Kordana and Mordalski, 2001

Slovakia, Czech Republic

Introductory and technological studies carried out in Slovakia were directed toward commercial cultivation of *Echinacea*. The studies focused on production technologies (Cerna et al., 1998) and the mechanization for commercial cultivation (Piszczalka et al., 1997) of the main three *Echinacea* species (Vaverková et al., 2001). More recent studies were reported in the Czech Republic concerning N-fertilization (Kolar et al., 1998).

Hungary

No specific research has been published in Hungary on any of the *Echinacea* species. The latest herb cultivation handbook includes detailed agrotechnical instructions on field cultivation methods of the three main species in Hungary (Praszna, 1993).

Bulgaria, Lithuania

In Bulgaria, a general description of *E. purpurea* was published by Evstatieva and Protich (1993). In Lithuania, biological observations and basic growing experiments have been carried out in Kaunas Botanical Garden since 1960. Biomass production, quantity, seed production, and quality of *Echinacea* have also been studied (Lapinskiene et al., 1999; Ragazinskiene and Lapinskiene, 2000; Skybitska et al., 2000).

Ukraine, Russia

E. purpurea was introduced into these areas at the end of the 20th century. The flowering biology of this species was studied by Porada and Rabinovich (1991). Mensova et al. (1987) studied the honey production of large-scale red coneflower plantations. The evaluation of *E. purpurea* as a possible medicinal plant was carried out by Porada (1992). Studies on seed biology have been reported recently (Babaeva et al., 1999).

Finland

The first agrobiological observations and climatic suitability of *E. purpurea* to the shorter and cooler Nordic growing periods were reported by Galambosi and Szebeni-Galambosi (1992). After winter tolerance observations were assessed, suitable organic growing methods were developed and submitted to the growers (Galambosi et al., 1994; Galambosi, 1995; Lääperi, 1995).

Norway

Based on Finnish data, Dragland et al. (1993), carried out agrobiological experiments in 1994 through 1997, aimed at introducing *E. purpurea* in Norway (Dragland, 1997). These results, together with the Finnish observations (Dragland and Galambosi, 1996) were submitted to growers in Norway.

Denmark

General cultural information published by Christensen et al. (2000) on the three *Echinacea* species was submitted to Danish growers.

Scotland

On the basis of grower interests in supplying raw material to local industrial companies, agrotechnical investigations commenced in South Scotland (Svoboda et al., 1996).

CULTIVATION AREAS IN EUROPE

Collecting data on the cultivation area of *Echinacea* in different European countries is difficult because the plants tend to be grown in small and scattered plots. Hence, there are no data available in national official statistics. For example, in Germany the total cultivation area for all *Echinacea* species was reported to be 178 ha in 1999, and only 85 ha in 2000. The cultivation area of *E. angustifolia* in France was reported to be 17 ha in 2000 (Aiello, 2002), and the total area for all species in 2002 was reported as 45 ha (Gicquiaud, 2002). On the other hand, cultivation regions are often located in close proximity of manufacturers of *Echinacea* products.

All available data are shown in Table 4.5. The estimated planted area of the three main *Echinacea* species in Europe is 250 to 300 ha. The biggest areas are in Germany, totaling 85 ha. A total of 30 to 50 ha are under cultivation in Italy, France, Poland, and Hungary; 13 to 20 ha in Sweden and Holland; and 3 to 5 ha in Switzerland, Spain, and Finland. *Echinacea* growing areas have increased significantly in Italy, from less than 10 ha in 1989 to 35 ha in 1999 (Vender, 2001).

The producers of *Echinacea* are quite various. There are farms and cooperatives producing raw materials only for commercial marketing or directly for processing industries. Some companies are only marketing *Echinacea* products while others are vertically integrated, involved from field cultivation, to processing (drying, extraction, product manufacture), to marketing.

In the recent European marketing survey book (Becker, 2000), *E. purpurea* is the most often used *Echinacea* species in Europe (Table 4.6). Company representative interviewees did provide separate reports on *E. pallida.* German companies dominate *Echinacea* product manufacturing in Europe.

TABLE 4.5
Cultivation Areas of *Echinacea* species in Europe

Country	Species	1999	2000	2001	2002	References
Germany	E	178				Kroth-Liersch, 2001
	EPu		60			Aiello, 2002
	EPa		20			Aiello, 2002
	EAn		5			Aiello, 2002
Italy	E	35				Vender, 2001
	EPa			30		Aiello, 2002
Austria	E			5–10		Aiello, 2002
France	EAn		17			Aiello, 2002
	EPu				40	Gicquiaud, 2002
	E				5	Gicquiaud, 2002
Poland	EPu				30–35	Kordana and Kucharski, 2002
Hungary	EPu				30	Tanito, 2002
The Netherlands	EPu				15–20	Mheen, 2002
Sweden	EPu				13	Langendorf, 2002
Finland	EPu				3	Galambosi, 2002
Norway	EPu				0.5	Dragland, 2002
Spain	EPu				3	Aiello, 2002
	EAn				1	Aiello, 2002
Switzerland	EPu				4	Rieser, 2002
	EAn				1	Gammeter, 2002

E = All *Echinacea* species; Epu = *E. purpurea*; Epa = *E. pallida*; Ean = *E. angustifolia*.

TABLE 4.6
Companies in Selected European Countries Specialized in *Echinacea* species Cultivation, Processing, and Trade

Country	Company	Activity	Coneflower, *Echinacea* sp.	Red coneflower, *E. purpurea*	Narrow-leaf coneflower, *E. angustifolia*
Belgium	Bioagrico BVBA	C,T	x	x	
France	Sicarappam	T	x		
	Tortay Freres (Ets)	T	x		
Hungary	Agroherba Kft	C,T		x	
Germany	Berghof Hräuter GmbH	C,P,T		x	x
	Agrimed Hessen W.V.	C,P	x		
	Heilpflanzen Sandorf GmbH & Co.KG	C,P	x	x	x
	Institut Drachenhaus	P	x		
	Madaus AG	C,P		x	
	Phytochem Referenzsubstanzen	P	x		
	Rieger-Hofmann GmbH	C,T	x		
	Jurgen Serr Herb-Service GmbH & Co.KG	P,T	x		
	Gärtnerei Winter	C	x		
Great Britain	Bioforce Ltd.	C	x		
	The National Herb Centre	C	x		
Italy	Chialva Nicolao s.a.s	P	x		
Norway	Norsk Oko-Urt BA	C,P,T	x		
Switzerland	Phytomed AG	C	x		

C = cultivation; P = processing; T = trade.

Source: Becker, P., 2000, *Business Guide, Medicinal and Spice Plants, Europe 2001*, Arznei- und Gewurzpflanze, Agrimedia Bergen/Dumme, Germany. With permission.

According to recent interviews carried out by Aiello (2002), the most important commercial product of *Echinacea* species is the dry root (Table 4.7). The prices of *E. purpurea* and *E. pallida* in European countries in 2002 ranged between 6 to 8 euros/kg, while that of *E. angustifolia* was about 10 to 15 Euros/kg. The prices of the fresh and dry herb products vary by country and by company.

DIFFERENT CULTIVATION AND PRODUCTION FORMS OF *ECHINACEA*

Decorative Perennial

Echinacea was introduced in Europe and cultivated first as a decorative perennial. Among the *Echinacea* species, *E. purpurea* is by far the best-known species. *E. pallida* and *E. angustifolia* have less ornamental value in Europe because their ligules have drooping characteristics.

As a perennial plant, its ornamental use is based on the large decorative and long-lasting inflorescens. Each plant has 10 to 30 flower stems and the large flowers are 6 to 18 cm in diameter, with 4- to 6-cm long ligules. Their color ranges from white to rose pink to red violet. After the flowering period, the dried elevated cones with ripened seeds have decorative value in the autumn garden as well. *Echinacea* species are also used in perennial plant borders (Hetman et al., 1996).

Cut Flower

In our Finnish cut-flower study, the longevity of the flowers in the field was 31 to 50 days and their full aesthetic flowering lasted indoors at room temperature for 10 to 12 days. The flower stems after cutting must be placed immediately, and remain continuously, in water to preserve longevity (Valo, 1995).

Nectar Production

According to a study carried out in Ukraine (Mensova et al., 1987), one *E. purpurea* plant, in years 2 and 3, developed 17 and 30 flowers, respectively. During the second and third years, one flower can produce 1.5 and 2.7 g of honey. Calculated based on plant density of 50 × 70 cm,

TABLE 4.7
Prices of *Echinacea* Products, Eurodollars/kg or CHF/kg, c. 2002

Country	Product	*E. purpurea*	*E. pallida*	*E. angustifolia*
Germany	Dry root	7	7	13
Italy	Dry root	6–8	6–8	10–15
France	Dry root			9.15–10.6
Spain	Dry root	6–9		
Switzerland	Dry root	22 CHF		
	Dry herb	15 CHF		
Finland	Fresh whole plant	1.5–1.8		
Poland	Dry root	1.5		
	Fresh root	0.3		

Source: Aiello, N., 2002, *ISAFA Communicazioni di Ricerca*, 1: 5–13. With permission.

one ha consists of 35,000 to 40,000 plants with 450,000 to 600,000 flowers. The calculated nectar production during the second and third years should be 25 to 75 kg/ha and 50 to 130 kg/ha, respectively. This result indicated that the flowers aged 3 or 4 years can be used for honey production as well.

Hydroponic Cultivation

Hydroponic cultivation techniques that are used generally for lettuce and fresh herb production in greenhouses (Both et al., 1999) provide new, totally controlled conditions for high-quality raw materials for industrial use of medicinal plants. Presently, these techniques are still in the experimental stage for *Echinacea*. The first experimental results have been recently reported in Canada on the cultivation of *E. angustifolia* using a floating-raft growing system (Pedneault et al., 2001).

In Vitro and Micropropagation Production of *Echinacea* Species

Micropropagation methods are the means of rapid clonal propagation of elite individual plants with unique ornamental or medical properties. Several studies have been carried out on the three main *Echinacea* species (Choffe et al., 2000; Harbage, 2000; Fulceri et al., 2001).

There are several publications describing the use of *in vitro* techniques for the production of *Echinacea* species (Coker and Camper, 2000; Krajewska-Patan et al., 1996; Sicha et al., 1991). The application of *in vitro* techniques to the production of *Echinacea* species provides an alternate source of plant material for extraction of active ingredients.

Regarding field production as a main source of the plant raw materials for industrial processing, the mass propagation method for commercial cultivation presently is either direct seed sowing or transplanting of seedlings. With respect to the end products of field cultivation, two main industrial utilization forms include the processing of the fresh plant material and of dried plant raw materials.

Fresh Raw Material

The fresh herb or root is sliced and pressed to obtain fresh plant juice, or the fresh plant material is frozen immediately after slicing and pressing. Other processing methods involve extraction of fresh raw materials by water, alcohol, or other organic solvents. Fresh raw material processing requires the close proximity of field and processing plant, and requires careful planning to avoid fermentation of processed material or contamination of active substances in fresh plant tissues.

Dried Raw Material

The most common and convenient method for preserving large quantities of raw plant materials is drying. The dominant part of the *Echinacea* raw materials used in national and international trade represents the dried forms of *Echinacea*.

The most common commercial items are leaves and whole aboveground parts: *E. purpurea herba, E. angustifolia herba, E. pallida herba*; and roots: *E. purpurea radix, E. angustifolia radix,* and *E. pallida radix*. Additionally, dried or soft extracts of the abovementioned raw materials are available on the market.

SPECIES AND CULTIVARS IN CULTIVATION

Species

As indicated in Table 4.5, *E. purpurea* cultivation represents nearly 65% of all *Echinacea* cultivated in Europe. It is the main species in Poland, Hungary, Netherlands, Sweden, and Finland, and the dominant species in Germany, Spain, and Switzerland.

The second most popular species, *E. pallida,* is cultivated in 25% of the total *Echinacea* cultivation area. Nearly all areas of *Echinacea* cultivation in Italy are of this species (30 ha), with 20 ha in Germany. *E. angustifolia* is cultivated in about 10% of the total *Echinacea* area in Europe. It is the principal species in France, and commercial plantations of this species are found in Germany as well.

Because of their great biological activity, but less advantageous agronomic features, *E. angustifolia* and *E. pallida* presently are under intensive agronomic study (Franke et al., 1997, 1999; Fulceri et al., 2001; Gatterer et al., 1999; Kordana and Mordalski, 2001; Sari et al., 1999).

Cultivars of *E. purpurea*

As mentioned previously, *Echinacea* species have been grown for their ornamental value in Europe since the 18th century. About 10 garden varieties of *E. purpurea* were in cultivation in Europe at least until 1960 (Bauer and Wagner, 1990).

E. purpurea has long been the focus of plant breeders who have found varieties within *E. purpurea*. *E. angustifolia* and *E. pallida* have no breed varieties defined thus far. Although *Echinacea* originated in North America, the purple coneflower species (*E. purpurea*) is probably better appreciated in Europe than in the United States as a garden ornamental plant (Foster, 1990). Many of the cultivars traded at present were developed in Europe. German plant breeders have focused on developing cultivars with ray flowers that do not droop. Consumers see drooping petals as being diseased or wilted. Presently, seed companies offer varieties in various shades of red and white for which origin or breeder is not always known.

Red-Colored Cultivars

Red-colored cultivars, which comprise the biggest group of ornamental varieties or cultivars, are listed below.

German origin: Abendsonne, Auslese, Leuchtstern (carmine or deep reddish rays, large flower heads), Roter Sonnenhut (1999, Chrestensen Co.), Schleissheim, Verbesserte Leuchtstern, Baumannshof, Magnus (spreading ray flowers, Klaus Jelitto Co.).

English origin: Bressingham Hybrid (dark cone with bright rose ray flowers), New Colewall Strain (compact, 15- to 18-cm wide head with greenish bronze center), Earliest of All (pink-purple ray flowers).

American origin: Bravado (lavender pink color, 1994, Park Seed Co.), Ovation Pink (from Well-Sweep Herb Farm, 1994).

Indeterminate origin: These are marketed by several seed companies and are known as The King (bright crimson rays), Bright Star (rosy red rays with maroon center), Pink Flamingo, Sombrero (crimson-purple rays), Robert Bloom (compact, purple red), Starlight (1999, Exotic Garden Co., Sweden), Hybrida, Rubinstern, Zwaan Kleve, and Benary.

White-Colored Cultivars

The smaller group of coneflower cultivars has white rays and a flower with a green disk and orange petals. The white-flowered variations all have been found in the wild (Foster, 1991). Cultivars that can be obtained from seed companies include Alba, White Prince, White King, White Lustre, and White Swan (a dwarf cultivar by Thompson & Morgan Co., USA, 1987).

Cultivars for Medicinal Purposes

In contrast to the numerous ornamental varieties or cultivars, only a few varieties exist for medicinal use. Because of industrial requirements for high quality and standardized components of the chemical constituents of plant raw materials, efforts have been made to develop these important cultivars.

In a study published in 1992 in Germany, biomass production and quality of 10 ornamental varieties were checked for medicinal purposes from 1986 to 1988 (Bomme et al., 1992a, 1992b). The varieties were Baumanshof, Benary, Hybrida, Leuchestern, Magnus, Rubinstern, Schleisheim, Verb Leuchtester, Zwaan Kleve, and White Lustre. Results showed that all these varieties/provenances were qualified for medicinal use. The proposed provenances follow:

Herb, 1 year: Schleisheim, Hybrida, and Vebesserte Leuchstern
Herb, 2 years: Hybrida and Verbesserte Leuchestern
Root, 2 years: Rubinstern
Root and herb: Rubinstern and Verbesserte Leuchestern

The great variability of echinacoside and cicoric acid contents of the commercially available *Echinacea* species was established in a study on medicinal properties (Schenk and Franke, 1996). In Hungary, a new selection of *E. purpurea*, cv. Indian, has been registered (Köck, 2001). The cultivar has 4- to 5-cm long, 0.5- to 0.6-cm wide purplish pink ray florets, leaning downward.

Because of common mistakes of plant identity and the adulteration of various *Echinacea* species, an analytical method has been developed for the fast chemical identification of *E. purpurea,* specifically the root components. By this method, the identification of a sample can be carried out within 2 minutes using near infrared reflectance spectroscopy (Laasonen et al., 2001).

ECOLOGY

In Europe, *Echinacea* grows well in the southern and central regions to southern Scandinavia without overwintering problems. However, central and northern Scandinavia seems to be the Nordic limit of its growth. The differences in ecologic requirements among species are not great.

Soil

In Poland, *Echinacea* is grown in soil with a pH of 6.5 to 7.2 (Mordalsky et al., 1994). In Finland, *E. purpurea* and *E. pallida,* but not *E. angustifolia,* were successfully cultivated in soil with a pH of 5.5 to 6.2 (Galambosi, 1995). *E. angustifolia* grows best in a more alkaline soil than the other *Echinacea* species, that is, a pH of approximately 8 appears suitable (Foster, 1991).

Echinacea is best suited to well-drained, moderately rich soil types, and an average sandy loam. Plants will not grow well in poorly drained soil. In Finland, overwintering problems have occurred in plain soils with long-term standing water (Galambosi et al., 1994). From the practical point of root harvest, sandy soil that can be easily washed from the roots is desirable. Soil with stones is undesirable.

Light

For optimal growth, *Echinacea* plants need full sun, but *E. purpurea* can tolerate up to 50% shade (Foster, 1991). *E. angustifolia* is an open plains plant that grows best in hot sun, whereas *E. purpurea* is a woodland plant that does not flourish under direct sun (Dey, 2000).

Water

Given their original natural habitats, it is not surprising that *Echinacea* species are well adapted to dry growing conditions in Europe. In cultivation, they are an exceptionally drought-tolerant species and stand up to such conditions better than any other perennial (Foster, 1991).

Differences in drought tolerance are based on their morphology. *E. purpurea* has large, moisture-containing leaves and hair-like roots. *E. purpurea* lives near forests, and requires higher moisture content in the soil than *E. angustifolia* and *E. pallida. E. angustifolia* and *E. pallida* have narrower

and hairy leaves, and deep taproots. Therefore they tolerate drought better. These differences must be kept in mind when choosing a locale for planting. In Hungary, growing *E. purpurea* in the northern parts of the country, where the precipitation is more regular, has been proposed (Praszna, 1993).

Temperature

In their native habitats, *Echinacea* species are frost-resistant and winter-hardy perennials, and they can tolerate −25°C to −40°C temperatures, provided there is snow cover (Chapman and Auge, 1994). In Europe, they safely overwinter in all parts of South and Central Europe. In 1995–1996 during a season of abnormally low winter temperatures (−21°C), good overwintering was reported even from Ayr, Scotland, at latitude 55° N (Svoboda et al., 1996).

Scandinavia is the northern limit of its commercial cultivation. *E. purpurea* overwinters in southern Sweden well, but not in Norway. According to observations in 43 Norwegian localities, temperature alterations were detrimental and overwintering was not safe (Dragland, 1997).

Echinacea overwintered quite safely in southern Finland at Nordic latitudes of 60° to 61°. However, from overwintering, problems occurred mainly after the first year of cultivation, when the seedlings were not well developed (weak growth, late transplanting time). During the 1984–1994 period, winter damage was observed four times after the first winters (Galambosi et al., 1994).

For successful *Echinacea* cultivation in the northern regions, mesoclimatic conditions (continuous snow cover, no standing water) and early transplanting of strong, well-developed seedlings are very important. Under optimal conditions, commercial cultivation has been practiced near the Arctic Circle at Oulunsalo (at the 65° N latitude) in Finland.

FIELD PRACTICES

Nutrient Supply

Information on nutrient requirements and fertilization of *Echinacea* species is very limited. In the early cultivation handbooks, the fertilization instructions are quite general. German writers have proposed mixing fertilizers in 100 to 200 kg/ha at ratios of N:P:K = 12:12:20 (Ebert, 1982) with additional compost between the rows every spring (Heeger, 1956).

In 1986, Bomme published the first growing instructions on *E. purpurea* and *E. pallida* with the following fertilization recommendation: nitrogen, 150 to 180 kg/ha; phosphorus, 70 to 100 kg/ha; and potassium, 220 to 250 kg/ha. This recommendation was followed by other cultivation handbooks in Europe. In Hungary, Praszna (1993) proposed the same doses with additional 30 tons/ha of manure in the previous autumn. In Poland, the first growing instructions proposed nitrogen, 60 to 80 kg/ha; phosphorus, 40 to 60 kg/ha; and potassium, 80 to 100 kg/ha (Mordalski et al., 1994).

According to Dachler and Pelzmann (1999), in soil with good conditions, phosphorus and potassium seem to be suitable in doses 70 and 150 kg/ha, respectively. The total quantity of nitrogen is 120 kg/ha, which is applied after sowing or transplanting, and after the first cut if a second cut is expected.

Detailed Experiments

Detailed studies on the fertilization of *Echinacea* started during the 1980s. The first fertilization data were published in northern Italy, where a fertilization trial was carried out using *E. pallida* in 1984–1985. The experimental area was in a mountain environment with acidic (pH = 4.95) and nonirrigated soil (Bezzi and Tessari, 1989). The applied nitrogen quantities were 0, 100, and 200 kg/ha and the phosphorus and potassium doses were 0 and 100 kg/ha, respectively. Bezzi and

Tessari (1989) found a positive effect of potassium on root production. The average root yields ranged between 1.1 to 1.3 tons/ha, while with higher doses of potassium, the root yields ranged between 1.5 to 1.9 tons/ha. The content of echinacoside — varying between 0.296% and 0.951% — was positively affected by the nitrogen and phosphorus.

The detailed nutrition requirements of the three *Echinacea* species were determined by German researchers (Bomme and Nast, 1998; Bomme and Wurzinger, 1990). According to their results, 1,000 kg of fresh *Echinacea* plant biomass contain 3 to 9 kg of nitrogen, 1 to 2 kg of phosphorus, and 4 to 8 kg of potassium. The quantities of these main elements vary with the species and plant parts (Table 4.8). The highest quantities of the main elements were extracted from *E. purpurea*, followed by *E. pallida,* and the lowest was from *E. angustifolia* (Table 4.9). In calculating the applied fertilization level, these figures have to be corrected for by the actual nutrient level of the soil, demonstrated by soil analyses. The calculated, appropriate phosphorus and potassium quantities for fertilization must be added in autumn and the appropriate nitrogen doses should be added separately in spring before transplanting, after the start of growth of young plants and after the first herb harvest.

In Poland, in a detailed experiment, the highest yield was obtained with N = 100, P_20_5 = 60, and K_2O = 100 kg/ha (Kordana et al., 1998). The effects of the fertilization on the dried root yield were evident during the second and third growing years (1.94 and 1.99 tons/ha, respectively), but decreased in the fourth year (1.35 tons/ha). The effect of the lack of nitrogen was more significant in the dry herb yields, which decreased linearly, that is, 8.38, 3.72 and 2.38 tons/ha, respectively. The total contents of the polyphenolic compounds ranged, in the herbs between 3.7% and 5.0% and in the roots from 1.6% to 3.5%, but the various fertilization levels had no effect on the contents of the polyphenolic compounds.

In another Polish experiment, the effect of two soil types on the yield was compared in pot conditions. The biomass yield depended on soil type and level of fertilization. The total biomass was higher on loamy soil by 64% to 71% in the first and second experimental years, but the contents of phenolic acids (chlorogenic, caffeic, and ferulic acids) were higher in sandy soil (Berbec et al., 1998).

Organic Fertilization

Much less data are available on organic fertilization of *Echinacea*. In Finland, composted and granulated chicken manure (Biolan) is used regularly in a dose of 2.5 tons/ha in organic cultivation

TABLE 4.8
Quantity of Principal Minerals in Biomass of *Echinacea* spp.

		Quantities of Mineral Elements (kg/1000 kg biomass)			
Species	**Biomass**	**N**	**P_2O_5**	**K_2O**	**MgO**
E. purpurea	Flowering shoot	4.4	1.3	8.1	1.4
	Root	4.6	1.4	5.0	1.4
E. pallida	Flowering shoot	3.1	1.0	4.5	1.1
	Root	5.8	1.2	5.2	0.7
E. angustifolia	Flowering shoot	5.6	1.2	8.2	1.4
	Root	9.5	2.0	4.5	1.0

Source: Bomme, U., 2000, *Technology of Field Cultivation for* Echinacea *species*, Bavarian State Research Centre for Agronomy, Freising, Germany (in German). With permission.

TABLE 4.9
Quantity of Mineral Elements Extracted from *Echinacea* spp.

Species	Harvested Fresh Biomass	Quantity (MT/ha)	Extracted Mineral Elements (kg/ha)			
			N	P_2O_5	K_2O	MgO
E. purpurea	Flowering shoot	30	133	38	248	44
	Root	15	69	21	76	21
	Total	45	202	59	324	65
E. pallida	Flowering shoot	30	44	31	134	32
	Root	15	88	18	79	11
	Total	45	132	49	213	43
E. angustifolia	Flowering shoot	5	28	6	41	7
	Root	2	19	4	9	2
	Total	7	47	10	50	9

Source: Bomme, U. and Nast, D., 1998, *Z. Arznei Gewurzpflanzen*, 3: 82–90. With permission.

(N:P:K = 4:1:2). Incorporating this quantity into soil before planting seems to be suitable for 2-year growth (Galambosi and Valo, 1995).

Weed Control

Chemical Weed Control

Although *Echinacea* grows in meadow ecosystems in wild places, it is not weed tolerant in cultivation. Therefore, although weed control is a very important factor throughout the entire cultivation period, it is especially important in the first year. In small cultivation areas, mechanical weeding is an ideal and easy way to keep populations free from weeds. However, in the case of larger, industrial production, chemical weed control becomes necessary, especially if direct sowing of seeds has been employed.

In Europe, registered herbicides exist only in Poland where several experiments were carried out for elaborating chemical weed control methods for *E. purpurea* (Kordana et al., 1996; Kucharski, 1997). Among 18 tested herbicides, three preparations have given good weed control alone or in combinations. Azogard (prometryn) in the dosage of 2.0 kg/ha provided good control of broadleaf weeds; Kerb 500 SC (propyzamid) in the dosage of 2 kg/ha controlled grass and broadleaf weeds; and Fusilade Super (fluazifop-P-butyl) in the dosage of 1.5 l/ha controlled grass weed. The residuals of these herbicides in the raw material were at a permissible level (Kucharski, 2000).

Weed Control in Organic Cultivation

Time-consuming manual weeding is one of the most significant factors in production cost and it is the main limiting factor in field size for cultivation. Mechanical weeding and the use of various mulches comprise two more practical methods for large-scale cultivation.

In larger-scale organic cultivation, the use of plastic mulch is common, since its spreading is mechanized at present for strawberry and cucumber cultivation. Plastic mulch can decrease the labor cost of weed control by 70% to 80% and produces a 114% increase in fresh plant weight (Galambosi and Szebeni-Galambosi, 1992). Cleaning rows between plastic mulch rows could be easily mechanized as well using regular lawnmowers. The heat accumulation in the soil under the plastic mulch in cooler areas is an additional advantage of this method. In warmer climates, use

of black plastic mulch could be a disadvantage since it retains heat. Consequently, growers need to prepare for irrigation.

Crop Protection

Thus far, only a few diseases and insect attacks have been observed in cultivation areas of *Echinacea*. Li (1998) concluded that plant disease does not seem to be a problem with *Echinacea* in North America. However, it is generally expected that during continuous cultivation, some new diseases and insects will occur. Only a few diseases have been reported in *Echinacea*: leaf spots caused by either *Cercospora rudbeckii* PK or *Septoria lepachydis* Ell&Ev, and root rot caused by *Phymatotrichum omnivorum* (Shear) Dug (Li, 1998). In Poland, Kucharski (1997) reported that *Alternaria alternata* was identified in *E. purpurea* cultivations.

For preventing disease infections, treatment of seeds before sowing is proposed in some countries. The preparations are Polyram-Combi for *E. purpurea* and *E. pallida,* in 0.2% concentration for 24 hours in Germany (Bomme, 2000). In Poland, Dithane M-45 or Penncozeb 75 WG in a dose of 3 to 5 g/kg of seed, or Dithane 455 SC or Penncozeb 455 SC in a dose of 4.5 ml/kg of seed are proposed (Kucharski, 1997). Kucharski (1997) listed the observed insects on *E. purpurea* in Poland: *Philenus spumarius, Phytomyza atricornis*, and *Liriomyza strigata*. In a coneflower growers manual, Polish experts proposed a wide range of insecticides as effective against these insects (Mordalski et al., 1994). The use of chemical preparations in plant cultivation is regulated by the authorities and varies among countries. Plant protection in organic cultivation is problematic at present.

Harvest and Yield

Harvest times of *Echinacea* depend on the propagation methods used, age of plantation, and species. The accumulation of biomass during the initial growing years is quite low. According to a Romanian study (Muntean et al., 1990, 1991), total fresh plant weights from transplanted seedlings were 414 g/plant of *E. purpurea* and 184 g/plant of *E. pallida*. The weights increased linearly to the third year, reaching 1422 g/plant fresh weight of *E. purpurea* and 1210 g/plant of *E. pallida* (Figure 4.1).

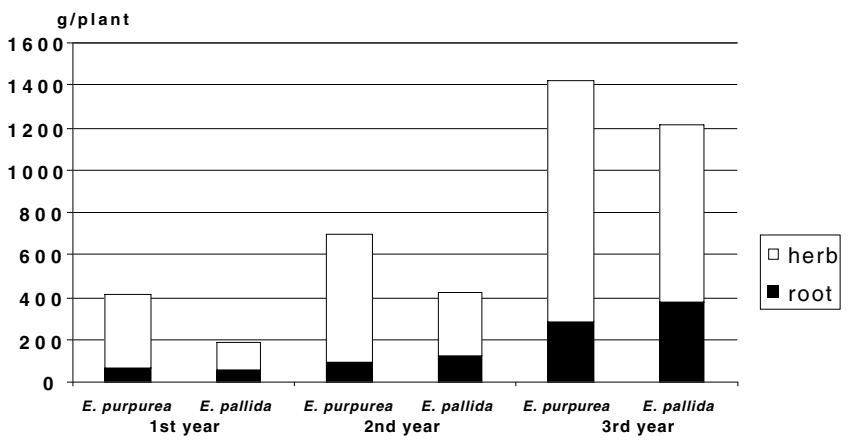

FIGURE 4.1 Total fresh weights of *Echinacea purpurea* and *Echinacea pallida* during 3 years' growth. (From Muntean et al., 1990, *Bul. Instit. Agric. Cluj-Napoca*, 44: 23–34, and Muntean et al., 1991, *Bul. USACN*, A-II, 45: 41–50. With permission.)

Good adaptation of *E. purpurea* to various climatic conditions shows that fresh plant biomasses were similar in Romania and Finland after the second year. The total fresh plant weight in Finland ranged between 547 to 870 g/plant (Galambosi, 1993), and in Romania, the average weight was 698 g/plant (Muntean et al, 1991).

According to German experiences and recommendations (Bomme, 2000), harvest possibilities of the main *Echinacea* species are summarized below.

Herb harvest, first year of cultivation: The optimum harvest time occurs when the main flowers are in full flowering. Of the three species, only *E. purpurea* flowers in the first growing year in central Europe. The other two species may have flowers at the end of the summer. In the northern part of Europe (e.g., Finland), in the case of transplanting in early June, there are no flowers at all during the first year. In the propagation years, flowering and harvesting times are generally in the early autumn: for *E. purpurea,* October; for *E. pallida,* the end of September or beginning of October; and for *E. angustifolia,* the end of September.

Second to fourth years of cultivation: After the propagation year, flowering of well-established populations starts earlier. The optimum harvest time occurs when the plants are in full flowering: for *E. purpurea,* the end of August or beginning of September; for *E. pallida,* the end of July or beginning of August; and for *E. angustifolia,* the end of July. For optimum contents of active ingredients, it is proposed to harvest as many full bloom flowers as possible. During autumn, a second harvest of the herb biomass may be achieved, but the proportions of the flowers then are generally lower. The stem height for cutting should not be lower than 10 cm aboveground. Lower cutting may result in poor overwintering and less growth in subsequent years. The times of the second harvest should not be too late in the year. In Poland, harvest in the second and third years is usually carried out between September 18 and 24, but in the fourth year, it is earlier, such as August 20 (Kordana et al., 1996). The harvest of herb biomass in smaller plots is carried out by hand, but on a larger scale, machinery is used.

Root Harvest

The root size of *E. pallida* and *E. angustifolia* is suitable for harvest beginning after the second growing year. According to Romanian and Polish experiences, root harvest during the end of the third year results in higher root yields. In the case of good growing conditions, root harvest of *E. purpurea* propagated from seedlings can occur in the first year.

The root harvest can be combined with the herb harvest as well. This means that before the root harvest, the herb could be utilized without harmful effects on root quality. The roots can be harvested in smaller areas by hand but on an industrial scale, machinery must be used. From the practical point of view, poor root harvests depend on improper root depth. Horizontally, the roots of *E. angustifolia* are concentrated in a region extending 150 mm on either side of the row. More than 90% of the roots of 2-year-old plants could be dug out from a depth of 27 cm. The roots of 3-year-old plants are deeper, since 78% and 92% of the total root yield are obtained from the 36- and 45-cm depth (Bantle et al., 2000).

Yields

The three *Echinacea* species differ in terms of biomass production and end yields. In general, *E. purpurea* is the highest-yield species, and the lowest-yield species is *E. angustifolia.* The yields of *E. pallida* range between these two. The differences in the dry herb and root yields in an Italian study after 3 cultivation years are presented in Figure 4.2.

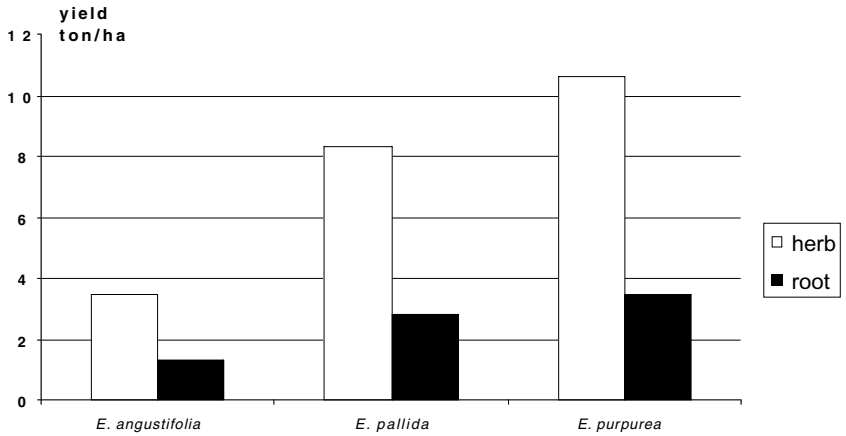

FIGURE 4.2 Dry herb and root yields of *Echinacea* spp. at Trento, northern Italy, after year 3 of cultivation. (From Aiello et al., 2002a, *ISAFA Communicationi di Ricerca,* 1: 15–27. With permission.)

Field cultivation yield depends on several factors such as age of plants, climate, place of cultivation, and cultivation methods used. On the basis of experimental and practical cultivation results, Bomme (2000) presented the possible yields of the three main species in Germany (Table 4.10). According to these results, in the optimal cases, the yields of *E. pallida* could be nearly as large as those of *E. purpurea*, producing 54 tons of fresh and 10 to 12 tons of dry herb/ha. The drying ratio between the fresh and dry herb yield of *E. purpurea* is generally 3.8 to 4.9:1, and for *E. pallida,* 4.0 to 5.5:1. Due to the higher dry matter content of the roots, the drying ratio of the root yields is lower, ranging between 2.6 and 4.0 to 1.

Seed Production

There is no specific report dealing with seed production of *Echinacea*. Only Lithuanian studies have reported seed productivity of *E. purpurea* (Skybitska et al., 2000). Collection of seeds is carried out during the second through the fourth years of plant age. Seeds must be collected from well-developed, healthy, and strong individual plants. One flower head contains 356 to 563 tubular flowers, but only 36% to 61% of them produce ripened seeds (Ragazinskiene and Lapinskiene, 2000).

The best seed collection time is when seeds are biologically ripe, 1 to 1.5 months after flowering, in August and September. During this time, the cones of the dry flowers are brownish in color. The best weather for seed production occurs when the air temperature is relatively high, with abundant sunny days and moderate quantities of rain. The harvest is carried out manually, selecting for the largest cones.

The harvested cones are kept in a dry place for a week and then the flower heads are crumbled mechanically. Larger quantities of flower heads are simultaneously crumbled by using a grain combine. The crumbled masses of seeds and stems then must be separated and seeds are finally cleaned by machine.

The separation of seeds from other parts of the flowers is somewhat difficult due to their similarity in weight and size. A laboratory separator for *E. purpurea* (Kamas Westrup LA-LS, Sweden) has a 4-mm hole size in its upper and middle screens, and 0.1 mm in the lower screen. The optimum speed of its motor is 390 rpm. The clean seeds are stored at a constant temperature in a moderate-humidity chamber.

TABLE 4.10
Fresh and Dry Yield Levels of *Echinacea* Species in Germany

Species	Year of cultivation	Yield/ha (MT)			
		Herb		Root	
		Fresh	Dry	Fresh	Dry
E. purpurea	1	10–37	2.4–8.5	–	–
	2–4	20–56	5.1–13.2	5.7–16.3	1.7–5.8
E. pallida	1	6–20	0.2–3.7	–	–
	2–4	11–54	2.5–11.9	3.9–17.0	1.1–5.7
E. angustifolia	1	1.7–4.0	0.4–0.9	–	–
	2–4	1.8–8.0	0.4–1.8	0.8–3.9	0.2–1.2

Source: Bomme, U., 2000, *Technology of Field Cultivation for Echinacea Species*, Bavarian State Research Centre for Agronomy, Freising, Germany (in German). With permission.

Mechanization

Mechanization is the main mechanism involved in large-scale cultivation of *Echinacea.* Without proper machinery, the cultivation areas and the quantity of the harvested raw materials remain small and costly, and labor intensive. Nevertheless, the grower must ensure that the machinery employed does not negatively affect the quality of the raw materials. The solutions used in mechanizing individual technological elements are quite variable, and reflect the machinery used in different countries, cultivation areas, and farms. Generally, machinery specific to medicinal plants does not exist nor has it been designed. Therefore, all existing machinery at the local level must be tested, and if necessary, adapted for the requirements of *Echinacea.* Machinery used in different aspects of cultivation is summarized below.

Soil preparation: plough, harrow, rotary harrow, rotary cultivator, tiller, bed ridger, bed lister
Fertilization: manure spreader, fertilizer spreader
Propagation: one-row manual seed driller, two- to five-row precision seed driller, greenhouse seed driller, pot filling and seeding line
Transplantation of seedlings: one- to four-row transplanter (for vegetables)
Crop protection: plant protection sprayer
Mechanical weeding: plastic film layer, interrow cultivator, row rotary cultivator, rotary weeder, rotary hoe, potato ridger, harrow, harrow comb
Herb harvest: self-loading trailer, flail, chopper, cutterbar unit, swather, rotary mower
Root harvest: carrot, sugarbeet harvester, potato elevator digger with windrowing attachment, root spinner, shaker digger, root washing machines, rotary drum washer, root cutting machines
Seed harvest: standing combine, crusher, threshing machine, seed dressing machine
Postharvest processing: chopper, cutter, press
Drying: dryers, batch dryer, conveyor belt dryer
Packaging: packaging machine

Quality Requirements

For *Echinacea* end products, there are pharmacopoeia standards or monographs at the national level; for example, in Germany such requirements are found in the *Deutsches Arzneibuch* (DAB).

In Europe, monographs issued by the European Scientific Cooperation on Phytotherapy (ESCOP) also include *E. purpurea radix, E. purpurea herba*, and *E. pallida radix* (ESCOP, 1997). However, there are no general, specific quality requirements for field cultivation. Medicinal companies have their own quality requirements; growers supply raw materials that must comply with them. These companies are mainly concerned with the authenticity of the plants cultivated, microbiological purity, and presence of pesticide residues and heavy metal contents of the raw materials.

Toward ensuring high-quality raw materials and end products for the medicinal industry, a strict compilation of regulations known as Good Manufacture Practice has been accepted in European Union countries. Significant efforts were then focused on the preparation of similar guidelines for field cultivation of medicinal plants as well, to ensure appropriate raw materials from the field (Franz, 1989). The results of efforts until 1989 were compiled by Franz (1989), and the final accepted guidelines of Good Agricultural Practices were published by Mathe and Franz (1999).

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Section III

The Chemistry of Genus Echinacea

5 Phytochemistry of the Genus *Echinacea*

Jeffrey B. Harborne and Christine A. Williams*

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INTRODUCTION

Echinacea is a small genus of the Compositae, tribe Heliantheae, containing six species (Shetler and Skog, 1978) that are all endemic to North America. Only three of the species — *E. angustifolia* DC., *E. purpurea* (L.) Moench, and *E. pallida* Nutt. — are used medicinally for their immunostimulatory properties (Bauer, 1998; Brevoort, 1996). The plant parts used include the rhizomes (usually termed roots in most references) of *E. angustifolia* and *E. pallida*, and less frequently of *E. purpurea*; the aerial parts of *E. purpurea*; and whole-plant homeopathic tinctures of *E. angustifolia* and *E. pallida*.

Phytochemical reports are restricted to these three medicinally important taxa. The identity of the commercially available plants of *E. angustifolia* from the botanic gardens of Europe that were used in analyses before *ca.* 1985 is suspect. Thus, more recent taxonomic determinations have revealed that the majority of these plants were in fact the taller more abundant species, *E. pallida*. Hence, most of the studies included here are post-1985. Four classes of compound are known to contribute to the immunostimulatory activity of *Echinacea* extracts: alkamides, glycoproteins, polysaccharides, and cinnamic acids. Alkamides (fatty acid amides) are characteristic rhizome components of *E. angustifolia* and the rhizomes and aerial parts of *E. purpurea*. Their absence from and the presence of polyacetylenes in rhizomes of *E. pallida* serve to distinguish this tissue from rhizome preparations of *E. angustifolia*. As in other members of the Heliantheae, the alkamides are mostly of the acetylenic type.

Glycoproteins, which induce cytokine production and show mutagenic activity, are other important rhizome constituents of both *E. angustifolia* and *E. purpurea*. Immunostimulatory polysaccharides present in the aerial parts and produced in tissue cultures of *E. purpurea* are of special interest

* Deceased

since one of the tissue culture products, an arabinogalactan, can now be produced on an industrial scale and is being considered for clinical trials. Hydrocarbons, mainly ketoalkenes and ketoalkynes (polyacetylenes), are further characteristic rhizome components of *E. pallida* with only small amounts of simple alkenes and esters present in rhizomes of the other two species.

Caffeoyl quinic and caffeoyl tartaric acid esters are the major cinnamic acid derivatives of all three species with each species having its own distinctive profile. For example, chicoric acid (2,3-dicaffeoyl tartaric acid), a major immunostimulatory rhizome component of *E. pallida* and of rhizomes and aerial parts of *E. purpurea*, is absent from rhizomes of *E. angustifolia*.

NITROGEN COMPOUNDS

Alkamides

Alkamides are fatty acid amides containing one or more double bonds that may be accompanied by up to three acetylenic linkages. Alkamides are characteristic constituents of the roots of *E. angustifolia* and the roots and aerial parts of *E. purpurea*. There is one old report of an alkamide named echinacein (12E, 4Z, 8E, 10E)-N-isobutyldodeca-2,4,8,10-tetraenamide) from roots of *E. angustifolia* (Jacobson, 1954, 1967), which has never been confirmed by others (Greger, 1988). The plant material used by Jacobson was most probably misidentified *E. pallida*, but even so Greger (1988) doubts that echinacein occurs in any *Echinacea* species. Alkamides had not been reported from the rhizomes of *E. pallida* until a recent study comparing the chemical components of different *Echinacea* species (Sloley et al., 2001), when they were found in small amounts in this tissue. Even so, the accumulation of alkamides as major rhizome constituents of *E. angustifolia* and *E. purpurea* can still be used to distinguish root powders of these plants from those of *E. pallida*. Polyacetylenes, on the other hand, have been found only in the roots of *E. pallida* (see section on polyacetylenes below), and so their presence or absence is another valuable diagnostic character for determining the composition of commercial root preparations.

As in other members of the Compositae, tribe Heliantheae, the alkamides reported from *E. angustifolia* and *E. purpurea* are mainly of the acetylenic type, together with a small number of purely olefinic structures (Table 5.1). Some 15 alkamides (1 to 15) (Figure 5.1) have been characterized in roots of *E. angustifolia* (Bauer et al., 1989a) (Table 5.1). These are derived mostly from undecanoic and dodecanoic acids and isobutylamide but also include trideca- (10), pentadeca- (6) and hexadecanoic acid (7) derivatives and two 2-methylbutylamides (4 and 5). The main constituents, the isomeric (2E, 4E, 8Z, 10E/Z)-N-isobutyldodeca-2,4,8,10-tetraenamides (14 and 15), were shown to display marked inhibitory activity *in vitro* in the 5-lipoxygenase and cyclo-oxygenase assays (Müller-Jakic et al., 1994), but showed only weak stimulation of phagocytosis (Bauer et al., 1989b). Four other 2,4-diene type alkamides (8,9,11,12) were detected but the remaining compounds (1 to 7 and 10) were all of the 2-monoene type, such as (E/Z)-N-isobutylundec-2-ene-8,10-diynamides (1 and 2).

In the roots of *E. purpurea*, ten alkamides (11 to 20) (Figure 5.1 and Figure 5.2) have been characterized, all of which are of the 2,4-diene type except for compound 19, trideca-2E,7Z-dien-10,12-diynic acid isobutylamide (Table 5.1). The major constituents were again compounds 14 and 15 (Bohlmann and Grenz, 1966; Bauer et al., 1988b) and three other *E. angustifolia* root constituents (11 to 13) were detected also in roots of *E. purpurea* (Bauer et al., 1988b).

The alkamides found in the aerial parts of *E. purpurea* are mainly of the same 2,4-diene type as in the roots with compounds 14 and 15 as the major components and five other root constituents (11, 13, 16, 17, and 19) (Figure 5.1 and Figure 5.2) as minor components. Three of five non-2,4-diene type alkamides, previously reported by Bohlmann and Hoffmann (1983) were not recorded in the more recent analysis of this tissue by Bauer et al. (1988c), who gave the reference but did not comment on the data. These compounds are (2E,7Z)-N-(2-methylbutyl)trideca-2,7-diene-10,12-

TABLE 5.1
Alkamides from *Echinacea* Species

Species	Part of Plant	Alkamide[b]	Reference
E. angustifolia	Rhizome[a]	(E)-N-isobutylundec-2-ene-8,10-diynamide (1)	Bauer et al., 1989a
		(Z)-N-isobutylundec-2-ene-8,10-diynamide (2)	Ditto
		(E)-N-isobutyldodec-2-ene-8,10-diynamide (3)	Ditto
		(Z)-N-(2-methylbutyl))undec-2-ene-8,10-diynamide (4)	Ditto
		(E)-N-(2-methylbutyl)undec-2-ene-8,10-diynamide (5)	Ditto
		(2E,9Z)-N-isobutylpentadeca-2,9-diene-12,14-diynamide (6)	Bolmann and Hoffmann, 1983; Bauer et al., 1989a
		(2E,9Z)-N-isobutylhexadeca-2,9-diene-12,14-diynamide (7)	Bauer et al., 1989a
		(2E,4Z,10Z)-N-isobutyldodeca-2,4,10-trien-8-ynamide (8)	Ditto
		(2E,4E)-N-isobutyldodeca-2,4-dienamide (9)	Ditto
		(2E,7Z)-N-isobutyltrideca-2,7-diene-10,12-diynamide (10)	Ditto
		(2E,4Z)-N-isobutylundeca-2,4-diene-8,10-diynamide (11)	Ditto
		(2Z,4E)-N-isobutylundeca-2,4-diene-8,10-diynamide (12)	Ditto
		(2E,4Z)-N-isobutyldodeca-2,4-diene-8,10-diynamide (13)	Ditto
		(2E,4E,8Z,10E)-N-isobutyldodeca-2,4,8,10-tetraenamide (14)	Ditto
		(2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (15)	Ditto
E. purpurea	Rhizome[a]	(2E,4Z)-N-isobutylundeca-2,4-diene-8,10-diynamide (11)	Bohlmann and Grenz, 1966; Bauer et al., 1988b
		(2E,4Z)-N-isobutyldodeca-2,4-diene-8,10-diynamide (13)	Ditto
		(2E,4E,8Z,10E)-N-isobutyldodeca-2,4,8,10-tetraenamide (14)	Ditto
		(2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (15)	Ditto
		(2Z,4E)-N-isobutylundeca-2,4-diene-8,10-diynamide (12)	Bauer et al., 1988b
		(2E,4E,10E)-N-isobutyldodeca-2,4,10-trien-8-ynamide (16)	Bauer et al., 1988b
		(2E,4E,8Z)-N-isobutyldodeca-2,4,8-trienamide (17)	Ditto
		(2E,4Z)-N-(2-methylbutyl)dodeca-2,4-diene-8,10-diynamide (18)	Ditto
		(2E,7Z)-N-isobutyltrideca-2,7-diene-10,12-diynamide (19)	Ditto
		(2E,4Z)-N-(2-methylbutyl)undeca-2,4-diene-8,10-diynamide (20)	Ditto
E. purpurea	Aerial parts	(2E,9Z)-N-isobutylpentadeca-2,9-diene-12,14-diynamide (6)	Bohlmann and Hoffmann, 1983; Bauer et al., 1989a
		(2E,7Z)-N-isobutyltrideca-2,7-diene-10,12-diynamide (19)	Bohlmann and Hoffmann, 1983

TABLE 5.1
Alkamides from *Echinacea* Species (continued)

		(2E,7Z)-N-(2-methylbutyl)trideca-2,7-diene-10,12-diynamide (21)	Ditto
		(2E,9Z)-N-(2-hydroxy-2-methylpropyl)pentadeca-2,9-diene-12,14-diynamide (22)	Ditto
		(2E,6E,8Z)-N-isobutyltrideca-2,6,8-triene-10,12-diynamide (23)	Ditto
		(2E,4Z)-N-isobutylundeca-2,4-diene-8,10-diynamide (11)	Bauer et al., 1988c
		(2E,4Z)-N-isobutyldodeca-2,4-diene-8,10-diynamide (13)	Ditto
		(2E,4E,8Z,10E)-N-isobutyldodeca-2,4,8,10-tetraenamide (14)	Ditto
		(2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10,-tetraenamide (15)	Ditto
		(2E,4E,10E)-N-isobutyldodeca-2,4,10-trien-8-ynamide (16)	Ditto
		(2E,4E,8Z)-N-isobutyldodeca-2,4,8-trienamide (17)	Ditto
E. atrorubens	Rhizome[a]	(2E,4Z)-N-isobutylundeca-2,4-diene-8,10-diynamide (11) and its (2E,4E) isomer (24)	Dietz and Bauer, 2001
		(E)-N-isobutylundec-2-ene-8,10-diynamide (1)	Ditto
		(2E,4Z)-N-isobutyldodeca-2,4-diene-8,10-diynamide (13)	Ditto
		(E)-N-isobutyldodec-2-ene-8,10-diynamide (3)	Ditto
		(2E,4Z,10Z)-N-isobutyldodeca-2,4,10-trien-8-ynamide (8)	Ditto
		(2E,7Z)-N-isobutyltrideca-2,7-diene-10,12-diynamide (10)	Ditto
		(2E,4E,8Z,10E)-N-isobutyldodeca-2,4,8,10-tetraenamide (14)	Ditto
		(2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (15)	Ditto
		(2E,4E,8Z)-N-isobutyldodeca-2,4,8-trienamide (17)	Ditto
		(2E,4E)-N-isobutyldodeca-2,4-dienamide (9)	Ditto
E. atrorubens	Aerial parts	(2E,4Z)-N-isobutylundeca-2,4-diene-8,10-diynamide (11)	Dietz and Bauer, 2001
		(2E,4E,8Z,10E)-N-isobutyldodeca-2,4,8,10-tetraenamide (14)	Ditto
		(2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (15)	Ditto
		(2E,4E)-N-isobutyldodeca,2,4-dienamide (9)	Ditto

[a] Referred to in most references as roots.
[b] For structures, see Figure 5.1 and Figure 5.2.

diynamide (21), (2E,9Z)-N-(2-hydroxy-2-methylpropyl)pentadeca-2,9-diene-12,14-diynamide (22) and (2E,6E,8Z)-N-isobutyltrideca-2,6,8-triene-10,12-diynamide (23) (Figure 5.2). According to Bohlmann and Hoffmann (1983), these compounds were difficult to isolate and were probably only present in small amounts.

In a recent study of another species, *E. atrorubens* Nutt., 11 alkamides were identified in the rhizome and 4 in the aerial parts (Table 5.1) (Figure 5.1 and Figure 5.2) (Dietz and Bauer, 2001).

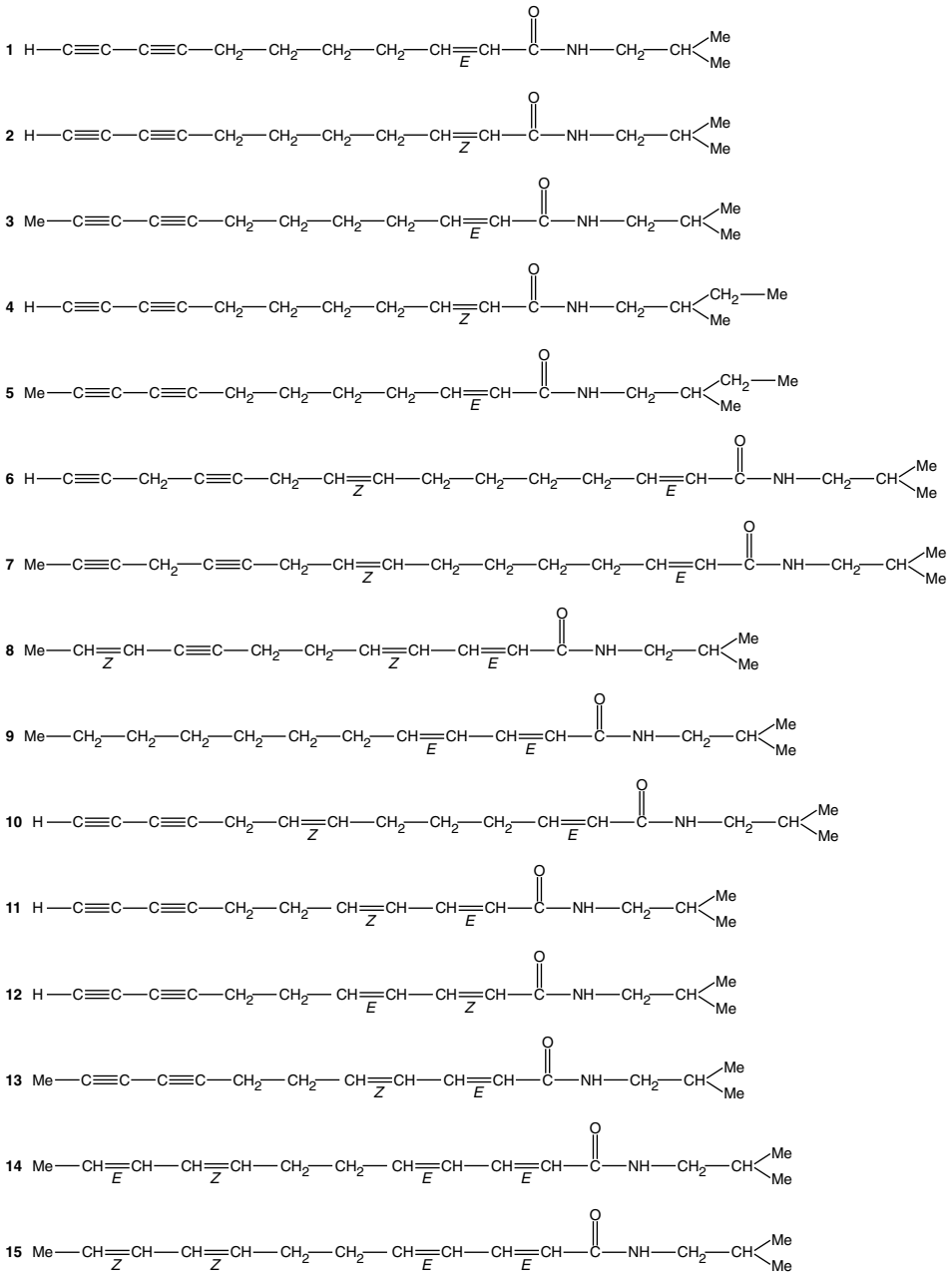


FIGURE 5.1 Alkamides found in *Echinacea* species.

The rhizomes proved to be very rich in these constituents, which included compounds previously found in rhizomes of both *E. purpurea* and *E. angustifolia*. Thus, the conjugated 2,4-dienoic acid amides 9, 11, 13, and 17 have all been reported from rhizomes of *E. purpurea*, while the monoenoic acid amides 1, 3, and 10 are typical of the rhizomes of *E. angustifolia.* The *trans*/*trans* isomer of 11, (2E,4E)-N-isobutylundeca-2,4-diene-8,10-diynamide (24) (Figure 5.2), was found for the first time in fresh rhizomes of *E. atrorubens* and was shown to increase in concentration after storage, suggesting that it is formed by decomposition of 11. As in *E. purpurea* and *E. angustifolia*, the

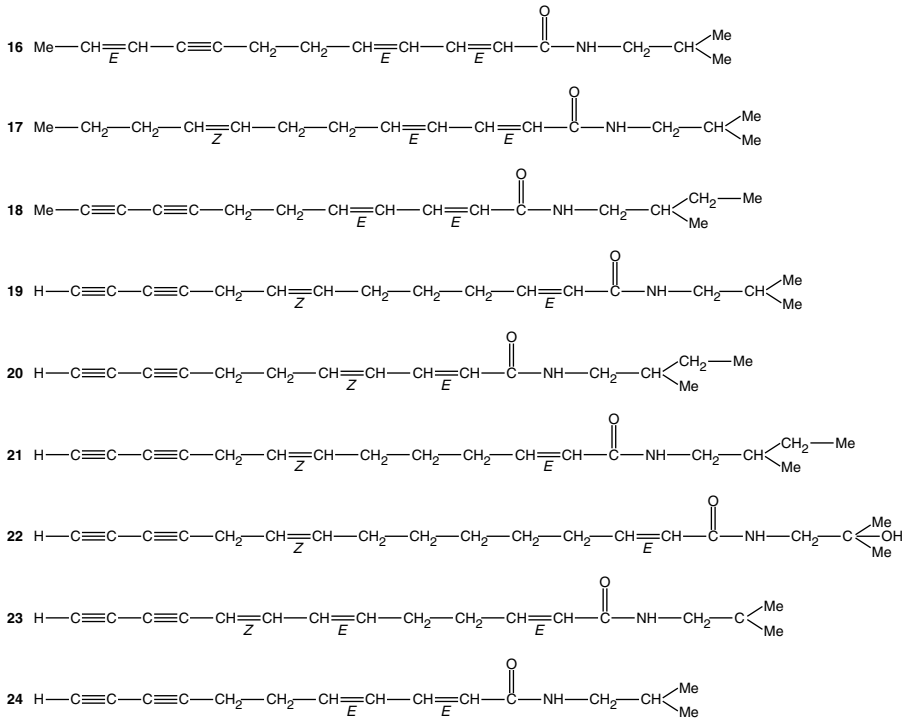


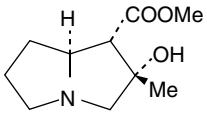
FIGURE 5.2 Further alkamides found in *Echinacea* species.

tetraene isomers 14 and 15 were the main alkamides in rhizomes of *E. atrorubens* with a concentration comparable to that in *E. angustifolia* rhizomes. However, the two species can be distinguished by the dominance of monoenoic amides in *E. angustifolia* rhizomes and conjugated 2,4-dienoic acid amides in *E. atrorubens* rhizomes. Also, polyacetylenes, which are characteristic constituents of *E. pallida* rhizomes, were not detected in those of *E. atrorubens* (Dietz and Bauer, 2001).

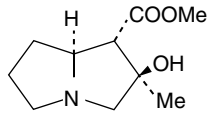
The concentration of alkamides in the aerial parts of *E. atrorubens* was much less than in the rhizomes. The major constituents were again the tetraene isomers 14 and 15 with a concentration about 15 times lower than in the rhizome. Compounds 9 and 11 were also detected in small amounts in this tissue (Dietz and Bauer, 2001).

Alkaloids

Lloyd (1897) reported the presence of a possible "colourless alkaloid" in roots of *E. angustifolia.* However, Heyl and Staley (1914) could not confirm the presence of true alkaloids but in the following year Heyl and Hart (1915) isolated betaine hydrochloride from this tissue. It is only recently that two pyrolizidine alkaloids, tussilagine (25) and isotussilagine (26) (Figure 5.3), were identified in whole plant extracts of *E. angustifolia* and *E. purpurea* (Röder et al., 1984). The major constituent, tussilagine (25), comprised about 15% of the crude extracts and was present as 0.006% of the dried root preparation of *E. angustifolia* (Britz-Kirstgen, 1985). Since neither tussilagin nor isotussilagin contain the 1,2-unsaturated necine ring, shown by Mattocks (1986) to be a requirement for the hepatotoxicity of pyrolizidine alkaloids, they should not cause liver damage. It is of taxonomic interest that it is the only report of this class of alkaloid in the tribe Heliantheae, where simple pyridine bases are normally the rule (Swain and Williams, 1977).



25. Tussilagine



26. Isotussilagin

FIGURE 5.3 Pyrrolizidine alkaloids found in *Echinacea* species.

GLYCOPROTEINS

Three glycoproteins with molecular weights of 17,000, 21,000, and 30,000, which contain approximately 3% protein, have been isolated from *E. angustifolia* and *E. purpurea* roots (Beuscher et al., 1987). The major components of the protein moiety were found to be aspartate, glycine, glutamate, and alanine while the main sugars were determined as arabinose (64% to 84%), galactose (1.9% to 5.3%), and glucosamines (6%). The roots of *E. angustifolia* and *E. purpurea* were shown to contain similar amounts of these glycoproteins using the ELISA method, which was developed specifically for their detection in *Echinacea* species (Egert and Beuscher, 1992). However, the roots of *E. pallida* contain significantly fewer glycoproteins than either of the above species (Beuscher et al., 1995). Glycoproteins have been implicated in the immunostimulatory activity of *Echinacea* extracts by inducing cytokine production and by their mitogenic activity (Bauer, 1993 and 1994).

POLYSACCHARIDES

Two polysaccharides, PS I and PS II, with immunostimulatory properties have been isolated from the aerial parts of *E. purpurea*. Their structures were determined as 4-*O*-methyl-glucuronoarabinoxylan (average MW 35,000) and an acidic arabinorhamnogalactan (MW 50,000), respectively, and they each showed significant activity in both *in vitro* and *in vivo* immunological assays (Wagner and Proksch, 1981; Stimpel et al., 1984; Proksch and Wagner, 1987). A crude polysaccharide fraction isolated from the roots of *E. purpurea* has not been analyzed in detail but appears to have a similar composition to that present in the aerial parts (Wagner et al., 1985).

The expense of obtaining pure polysaccharides from plant extracts and the difficulty of obtaining reproducible activities led to the use of tissue culture for their isolation. Three homogeneous polysaccharides, two neutral fucogalactoxyloglucans with molecular weights of 10,000 and 25,000, and an acidic arabinogalactan (MW 75,000) were isolated from cell cultures of *E. purpurea* (Wagner et al., 1988). Wagner et al. (1988) found that the fucogalactoxyloglucan with MW of 25,000 enhanced phagocytosis both *in vitro* and *in vivo,* while the arabinogalactan specifically stimulated macrophages to excrete the tumor necrosis factor (TNF). The acidic arabinorhamnogalactan from *E. purpurea* is now produced biotechnologically on an industrial scale and may be considered for clinical trials (Wagner et al., 1999). According to Stephen (1983), it can be classified as an arabinogalactan of type II with a (1→3)-linked β-D-galactan backbone, which is probably covalently attached to a rhamnogalactan chain and an arabinan chain. The structures of the polysaccharides produced by tissue culture differ from those in the aerial parts because they are primary wall components of the cultured cells. Thus, the polysaccharide obtained from plants of *E. purpurea* shows little similarity to that produced by cell culture.

A pectin-like polysaccharide has been isolated from the expressed sap and a xyloglucan (molecular weight 79,500) from the leaves and stems of *E. purpurea* (Stuppner, 1985). The roots of *E. angustifolia* are reported to have a 5.9% inulin content (Heyl and Staley, 1914). Bonadeo et al. (1971) isolated a polysaccharide mixture composed largely of an acidic mucopolysaccharide that they termed "echinacin B," which showed weak antihyaluronidase activity. Wagner et al. (1985)

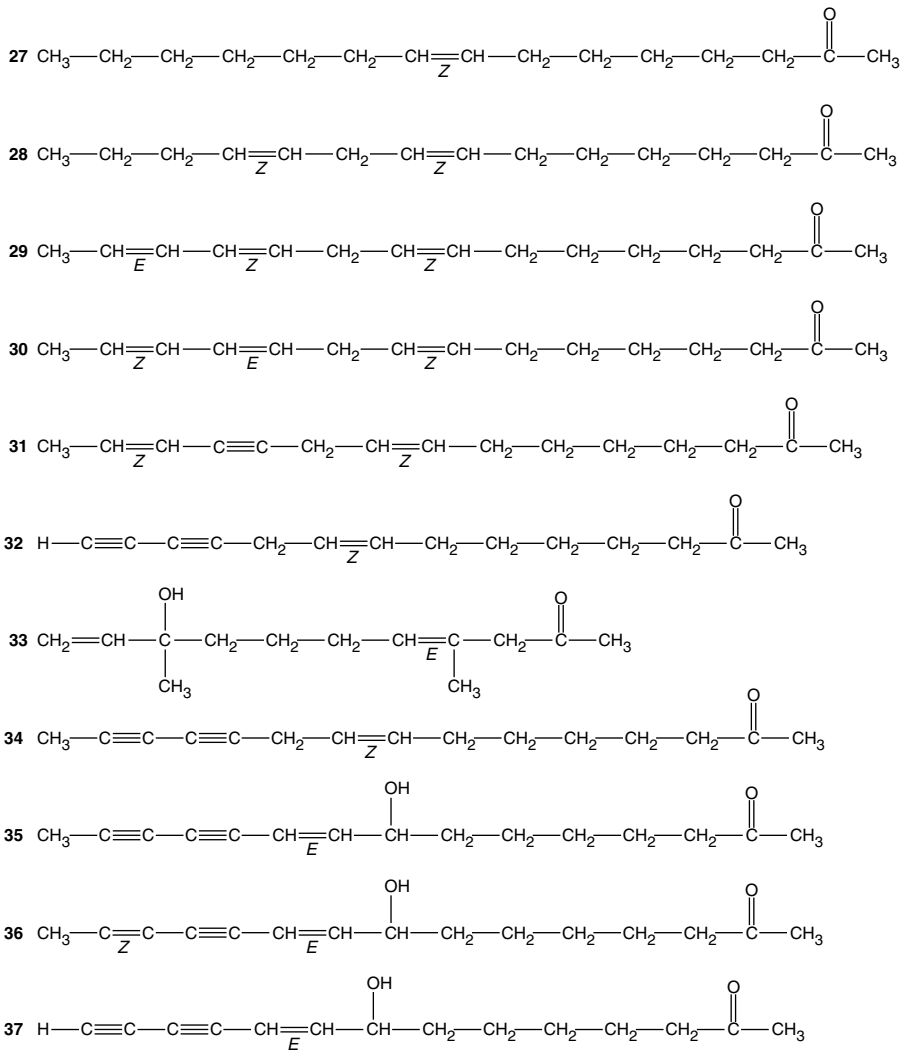


FIGURE 5.4 Ketoalkenes and a ketoalkyne found in *Echinacea* species.

have more recently isolated a crude polysaccharide fraction from the roots of *E. angustifolia* but did not complete the characterization of the individual components.

HYDROCARBONS

Hydrocarbons are characteristic root constituents of *E. pallida*, where some 11 derivatives, mainly ketoalkenes and ketoalkynes (polyacetylenes) have been identified (Figure 5.4). The major components (Table 5.2) are the ketoalkenes — (Z)-pentadec-8-en-2-one (27), (8Z,11Z)-pentadeca-8,11-dien-2-one (28) (Heinzer et al., 1988; Bauer et al., 1988a), (8Z,11Z,13E)-pentadeca-8,11,13-trien-2-one (29), and (8Z,11E,13Z)-pentadeca-8,11,13-trien-2-one (30) (Schulte et al., 1967; Khan, 1987; Bauer et al., 1988a) — and the ketoalkenynes — (8Z,13Z)-pentadeca-8,13-dien-11-yn-2-one (31), (Z)-tetradeca-8-diene-11,13-diyn-2-one (32) (Heinzer et al., 1988; Bauer et al., 1988a), and (*Z*)-pentadeca-8-ene-11,13-diyn-2-one (34) (Schulte et al., 1967; Khan, 1987; Bauer et al., 1988a). Two

TABLE 5.2
Hydrocarbons from Rhizomes[a] of *Echinacea* Species

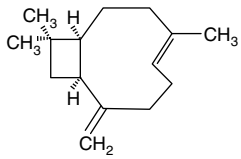
Species	Hydrocarbon[b]	Reference
E. angustifolia	Dodeca-2,4-dien-1-yl isovalerate	Heinzer et al., 1988
	(Z)-Pentadeca-1,8-diene	Voaden and Jacobson, 1972
	Pentadec-1-ene	
E. pallida	(Z)-Pentadec-8-en-2-one (27)	Schulte et al., 1967
	(Z)-Pentadeca-1,8-diene	Voaden and Jacobson, 1972
	Pentadec-1-ene	
	(8Z,11Z)-Pentadeca-8,11-dien-2-one (28)	Heinzer et al., 1988
	(8Z,13Z)-Pentadeca-8,13-dien-11-yn-2-one (31)	Bauer et al., 1988a
	(Z)-Tetradeca-8-diene-11,13-diyn-2-one (32)	
	(E)-10-Hydroxy-4,10-dimethyldodeca-4,11-dien-2-one (echinolone) (33)	Jacobson et al., 1975
	(Z)-Pentadec-8-ene-11,13-diyn-2-one (34)	Schulte et al., 1967
	(8Z,11Z,13E)-Pentadeca-8,11,13-trien-2-one (29)	Bauer et al., 1988a
	(8Z,11E,13Z)-Pentadeca-8,11,13-trien-2-one (30)	Khan, 1987
E. purpurea	Dodeca-2,4-dien-1-yl isovalerate	Heinzer et al., 1988

[a] Referred to as roots in most references.
[b] For structures, see Figure 5.4.

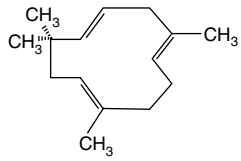
other major root constituents of *E. pallida*, the simple alkenes pentadec-1-ene (Voaden et al., 1972; Oniga et al., 1997) and (*Z*)-pentadeca-1,8-diene (Schulte et al., 1967) have been detected in roots of *E. angustifolia* (Voaden et al., 1972) (Table 5.2). The only hydrocarbon reported from roots of *E. purpurea* is an ester, dodeca-2,4-dien-1-yl isovalerate, which is present also in roots of *E. angustifolia* (Heinzer et al., 1988) (Table 5.2). The absence of polyacetylenes from roots of both *E. angustifolia* and *E. purpurea* provides a useful means of differentiating root preparations of *E. pallida* from those of these other two species. By this means, Bauer et al. (1988a) have proved that commercially produced root preparations of *E. pallida* are commonly contaminated with *E. angustifolia*. This suggests that some early reports of hydrocarbons in *E. angustifolia* roots are due to misidentification of/or contamination with *E. pallida*. One such paper describes a tentative structure of (E)-10-hydroxy-4,10-dimethyl-4,11-dodecadiene-2-one (33) for an insect growth regulator polyine, named echinolone, which induces strong juvenilizing effects in the yellow mealworm, *Tenebrio molitor* L. (Jacobson et al., 1975). The polyacetylenes in *E. pallida* roots are very susceptible to autoxidation to 8-hydroxy-9-ene derivatives. Three such artifacts have been identified: (9E)-8-hydroxypentadeca-9-ene-11,13-diyn-2-one (35), (9E, 13Z)-8-hydroxypentadeca-9,13-dien-11-yn-2-one (36), and (9E)-8-hydroxytetradeca-9-ene-11,13-diyn-2-one (37) (Figure 5.4) (Bauer et al., 1988a).

Essential Oils

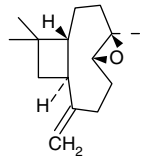
The roots of *E. purpurea* have been reported to contain up to 0.2% essential oil (Bauer, 1999). The major components were found to be caryophyllene (38) (2.1%), humulene (39) (0.6%), and caryophyllene epoxide (40) (1.3%) (Figure 5.5) (Becker, 1982; Martin, 1985). The flowering aerial parts of this plant were reported to contain less than 0.1% essential oil. The constituents identified include borneol (41), bornyl acetate (42), germacrene D (43), and caryophyllene and its epoxide (Figure 5.5), which were present also in aerial parts of *E. pallida* and *E. angustifolia* (Bauer, 1999). Schulthess et al. (1991) analyzed the essential oil of the achenes of *E. purpurea* and identified α-



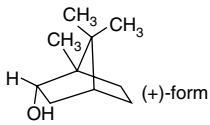
38. Caryophyllene



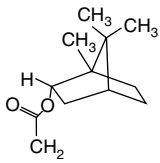
39. Humulene



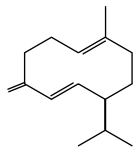
40. Caryophyllene epoxide



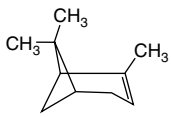
41. Borneol



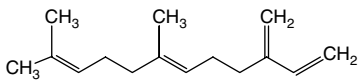
42. Bornyl acetate



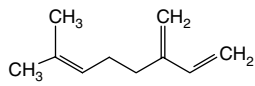
43. Germacrene D



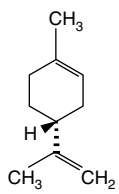
44. α-Pinene



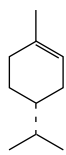
45. β-Farnesene



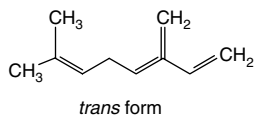
46. Myrcene



47. Limonene

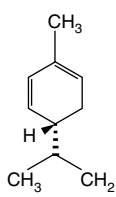


48. Carvomenthene

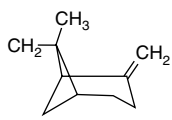


trans form

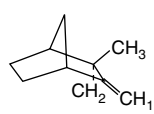
49. β-Ocimene



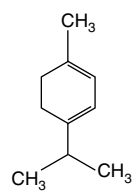
50. α-Phellandrene



51. β-Pinene



52. Camphene



53. Terpinene

FIGURE 5.5 Essential oils found in *Echinacea* species.

pinene (44), β-farnesene (45), myrcene (46), limonene (47), carvomenthene (48), caryophyllene, and germacrene D (Figure 5.5). Mazzi and Cottrell (1999) analyzed the headspace volatile components of roots, stems, leaves, and flowers of *E. angustifolia*, *E. pallida*, and *E. purpurea* using capillary GC/MS and identified over 70 compounds. The main essential oils in all the plant tissues, irrespective of species, were camphene, β-pinene, and limonene, together with other volatiles such as acetaldehyde, dimethyl sulphide, and hexanal. The aerial parts also contained β-myrcene (46), α-pinene (44), and *trans*-ocimene (49) plus 3-hexen-1-ol and 2-methyl-4-pentenal. α-Phellandrene (50) (Figure 5.5) was found to be the major essential oil component of the rhizomes of *E. purpurea* and *E. angustifolia*, but was not present in rhizomes of *E. pallida.* Aldehydes, especially butanals and propanals, made up 41% to 57% of the head space of rhizome tissue, 19% to 29% of the leaf head space, and only 6% to 14% of the head space of the flowers and stems of the three species. Terpenoids, including α-pinene (44), β-pinene (51), β-myrcene (46), ocimene (49), limonene (47), camphene (52), and terpinene (53) (Figure 5.5), made up 81% to 91% of the head space of flowers and stems and 46% to 58% of the head space of leaf tissue, but only 6% to 21% of the rhizome head space.

Flavonoids

There are only three reports of flavonoids from *Echinacea* species. Cheminat et al. (1989) identified the major anthocyanin pigments in the flowers of *E. purpurea* and *E. pallida* as cyanidin 3-glucoside (54) and cyanidin 3-(6"-malonylglucoside) (55) (Figure 5.6). Anthocyanin-producing callus and suspension cultures were derived from the stem of *E. purpurea* by Cheminat et al. (1989). Three anthocyanins were isolated from these suspension cultures, cyanidin 3-glucoside and two further acylated cyanindin glycosides that were not fully characterized (Cheminat et al., 1989). The only other report derives from unpublished data in a doctoral thesis of trace amounts of quercetin and

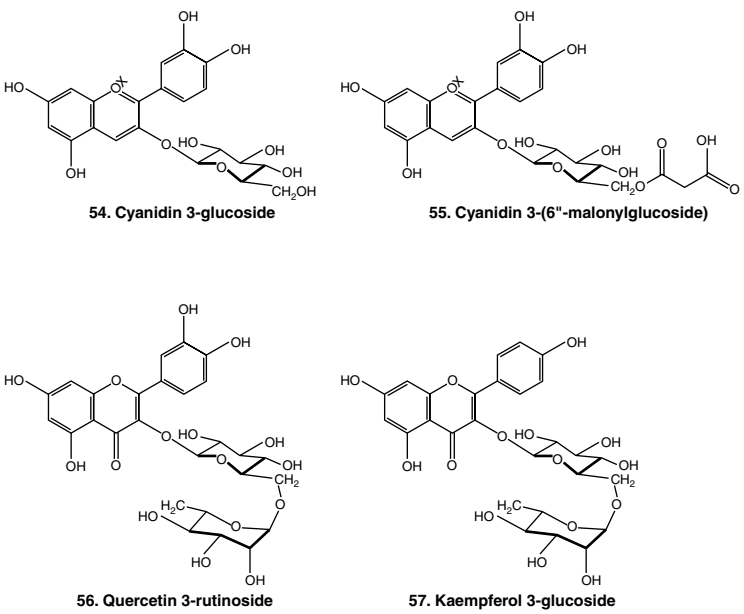


FIGURE 5.6 Flavonoids found in *Echinacea* species.

TABLE 5.3
Distribution of Caffeic Acid Conjugates in Flowers, Leaves, and Rhizomes[a] of *Echinacea pallida*

Caffeic Acid Conjugates[b]	Flower	Leaf	Rhizome[a]
Chlorogenic acid (5-O-caffeoylquinic acid) (66)	++	++	++
3,5-O-Dicaffeoylquinic acid (61)	++	+	+
4,5-O-Dicaffeoylquinic acid (62)	++	+	+
Chicoric acid (2,3-O-dicaffeoyltartaric acid) (58)	+++	+++	+++
2-O-Caffeoyl-3-O-feruloyltartaric acid (67)	?	++	?
Caftaric acid (2-O-caffeoyltartaric acid) (65)	++	++	++
2-O-Caffeoyl-3-O-5-[α-carboxy-β-(3,4-dihydroxyphenyl)ethyl]caffeoyltartaric acid (75)	–	++	–
2,3-O-Di-5-[α-carboxy-β-(3,4-dihydroxyphenyl)ethyl]caffeoyltartaric acid (74)	–	++	–
β-(3,4-Dihydroxyphenyl)-ethyl-O-4-O-caffeoyl-β-D-glucopyranoside (desrhamnosylverbascoside) (64)	++	+	+
β-(3,4-Dihydroxyphenyl)ethyl-O-α-L-rhamnopyranosyl(1→3)-4-O-caffeoyl-β-D-glucopyranoside (verbascoside) (63)	++	+	+
β-(3,4-Dihydroxyphenyl)ethyl-O-α-L-rhamnopyranosyl(1→3)-β-D-glucopyranoside(1→6)-4-O-caffeoyl-β-D-glucopyranoside (echinacoside) (59)	++	+	+++
β-(3,4-Dihydroxyphenyl)ethyl-O-α-L-rhamnopyranosyl(1→3)(6-O-caffeoyl-β-D-glucopyranosyl(1→6)-4-O-caffeoyl-β-D-glucopyranoside (6-O-caffeoylechinacoside) (60)	–	–	++

[a] Referred to as roots in source.

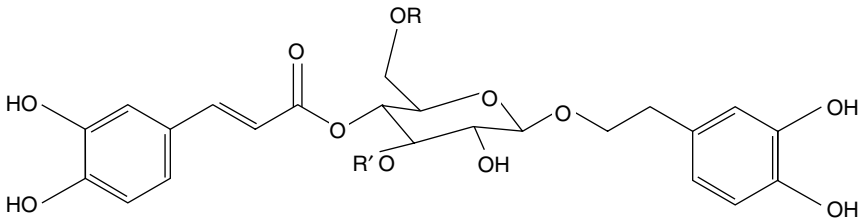
[b] For structures, see Figure 5.7 through Figure 5.9.

Source: Cheminat, A., et al., 1988, *Phytochemistry*, 27, 2787–2794. With permission.

kaempferol glycosides, including the 3-rutinosides (the rhamnosyl(1→6)glucosides, 56, 57) in the aerial parts of *E. purpurea* (Malonga-Makosi, 1983).

Cinnamic Acids

Caffeoylquinic and caffeolytartaric esters are characteristic phenolic constituents of *E. angustifolia, E. purpurea*, and *E. pallida*. The number and variety of structures present can be used to distinguish between both fresh and commercial preparations of these taxa. Some 12 caffeoyl conjugates, which have been variously identified in flowers, leaves, and roots of *E. pallida,* are listed in Table 5.3 (Cheminat et al., 1988), and the structures are given in Figure 5.7 through Figure 5.9. This species is particularly rich in these constituents and is unusual in that the different plant parts differ markedly in their caffeoyl conjugate profiles. Thus, chicoric acid (58) is the major component in all three organs, while echinacoside (59) is present in an equally high concentration in the roots but in only moderate amounts and trace amounts in flowers and leaves, respectively. 6-Caffeoylechinacoside (60) occurs only in the roots as a minor constituent, while flowers are characterized by substantial amounts of 3,5-O-dicaffeoylquinic acid (61), 4,5-O-dicaffeoylquinic acid (62), verbascoside (63), and desrhamnosylverbascoside (64). According to Cheminat et al. (1988), all the plant parts of *E. purpurea* had similar profiles with chicoric acid as the major constituent, caftaric acid (2-caffeoyl-tartaric acid) (65) present in significant amounts, and chlorogenic acid (66) as a minor component. However, according to Bauer et al. (1988c) and Remiger (1989), chicoric acid is more abundant in the flowers, especially the ligules, with much less in the leaves and stems. Also the leaves have been shown to additionally contain cichoric acid methyl ester (67), 2-caffeoyl-3-feruloyltartaric acid (68), 2,3-diferuloyltartaric acid (69) (Becker and Hsieh, 1985), 2-feruloyltartaric acid (70),



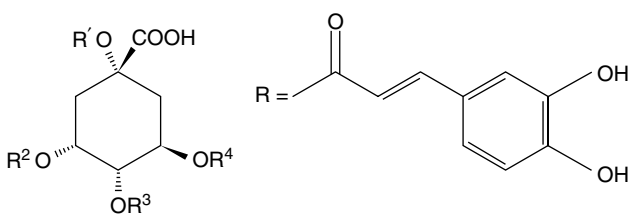
59 R = Glc (l→ 6), R′ = Rha (l→ 3)

60 R = 6-*o*- Caffeoyl Glc(l→ 6), R′ = Rha (l→ 3)

63 R = H, R′ = Rha (l→ 3)

64 R = R′ = H

FIGURE 5.7 Some caffeic acid sugar esters found in *Echinacea* species.



61 $R' = R^3 = H,\ R^2 = R^4 = R$

62 $R' = R^2 = H,\ R^3 = R^4 = R$

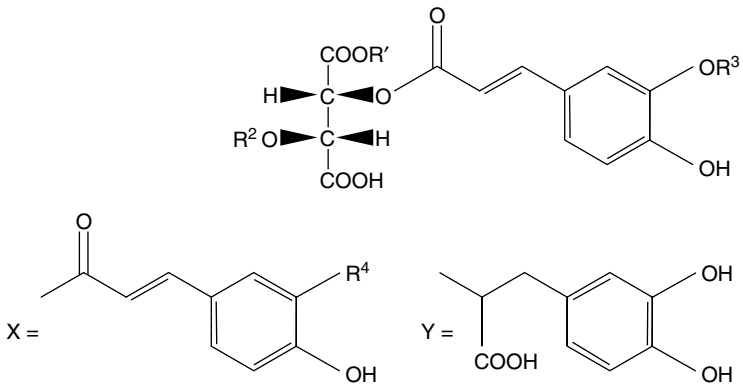
66 $R' = R^2 = R^3 = H,\ R^4 = R$

72 $R' = R^2 = R,\ R^3 = R^4 = H$

73 $R' = R^4 = R,\ R^2 = R^3 = H$

FIGURE 5.8 Quinic acid esters of caffeic acid found in *Echinacea* species.

and 2-caffeoyl-3- *p*-coumaroyltartaric acid (71) (Soicke et al., 1988). The major caffeoyl constituent of the roots of *E. angustifolia* was identified as echinacoside (59) (Stoll et al., 1950), and the absence of chicoric acid and the presence of cynarine (1,3-dicaffeoylcaffeoyl quinic acid) (72) and 1,5-dicaffeoylquinic acid (73) in the roots distinguish this species from both *E. purpurea* and *E.*



58 R′ = R^3 = H, R^2 = X, R^4 = OH

65 R′ = R^2 = R^3 = H

67 R′ = Me, R^2 = X, R^3 = H, R^4 = OH

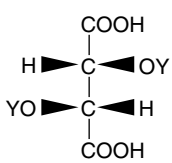
68 R′ = R^3 = H, R^2 = X, R^4 = OMe

69 R′ = H, R^2 = X, R^3 = Me, R^4 = OMe

70 R′ = R^2 = H, R^3 = Me

71 R′ = R^3 = R^4 = H, R^2 = X

74



75 R′ = R^3 = H, R^2 = Y

FIGURE 5.9 Tartaric acid esters of cinnamic acids found in *Echinacea* species.

pallida. Similarly, the absence of echinacoside distinguishes *E. purpurea* from *E. pallida* and *E. angustifolia*.

Cichoric acid (58) has been shown to exhibit phagostimulatory activity *in vitro*, whereas echinacoside and caftaric acid (65) did not (Bauer et al., 1989b). Cichoric acid was found also to inhibit hyaluronidase activity (Facino et al., 1993). Both cichoric acid and echinacoside have been demonstrated to dose-dependently protect the free radical–induced degradation of Type III collagen by a reactive scavenging effect, suggesting they could help prevent UV-B damage to the skin (Facino et al., 1995).

ACKNOWLEDGMENTS

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6 The Chemistry of Antioxidant Constituents of *Echinacea*

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INTRODUCTION

Living organisms are exposed to 20.93% oxygen in air, which contributes to a dependency on biological oxidation reactions as a source of energy required for natural growth and metabolism. Under normal conditions, 2% of the oxygen consumed by mitochondria is incompletely reduced, thus resulting in the production of oxygen radicals (Boveris and Chance, 1973). If mitochondria electron transport is compromised, the percentage of oxygen incompletely reduced will increase. There is a potential, therefore, for a relatively small percentage of oxygen derived from systemic respiration to be transformed to bioactive agents, which in turn can ultimately lead to peroxidation reactions of cellular constituents that are composed of nucleic acids, lipids, proteins, and carbohydrates. In addition to endogenously derived reactive oxygen species (ROS), living organisms can also be exposed to ROS derived from exogenous sources such as environmental exposure to ultraviolet light, smoking, and pollution. Besides oxygen, nitrogen also has a central role in biology, since nitrogen generates a series of free radical or nonradical species (reactive nitrogen species, RNS). Common reactive oxygen and nitrogen species include superoxide anion ($O^{\bullet -}_2$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO^{\bullet}_2$), and peroxynitrite ($ONOO^-$) (Halliwell and Aruomoa, 1997). A list of reactive oxygen and nitrogen species is shown in Table 6.1.

In addition to posing a health risk through potential peroxidation reactions that affect cell viability, some ROS such as superoxide radical and hydrogen peroxide are also involved in important reactions that ensure optimal cellular function (e.g., phagocytosis and cell signaling). Under normal conditions, the body has a mechanism for balancing intracellular and/or extracellular events, which ultimately contribute to the generation of ROS, termed "oxidative stress." This condition will involve both optimal activity of various endogenous tissue antioxidant enzyme systems (e.g., superoxide dismutase, glutathione peroxidase, catalase) (Yuan et al., 1996) and the presence of nonenzymatic antioxidants that reside in close proximity to the cellular site where oxidation reactions occur (e.g.,

TABLE 6.1
Sources of Reactive[a] Oxygen and Nitrogen Species

Name	Symbol	Origin	Radical/Nonradical
Superoxide radical	$O_2^{\bullet -}$	$O_2 + e^-$	Radical
Hydroxyl radical	$^{\bullet}OH$	H_2O, H_2O_2	Radical
Alkoxyl radical	$LO^{\bullet}$	LOOH, $LOO^{\bullet}$	Radical
Peroxyl radical	$LOO^{\bullet}$	LOOH, $L^{\bullet}+O_2$	Radical
Hydrogen peroxide	H_2O_2	$^{\bullet}O_2$	Nonradical
Hydroperoxide	LOOH	$LOO^{\bullet}$, $^1O_2$	Nonradical
Singlet oxygen	$^1O_2$	Photo-oxidation, $^3O_2$	Nonradical
Nitric oxide	$NO^{\bullet}$	Arginine	Radical
Nitric dioxide	$NO_2^{\bullet}$	$LOO^{\bullet}{}_2$ + NO	Radical
Peroxynitrite	$ONOO^-$	$O_2^{\bullet -}$ + $NO^{\bullet}$	Nonradical

[a] Reactivity of ROS and NOS varies and H_2O_2, $NO^{\bullet}$, and $O^{\bullet -}{}_2$ react quickly and with specificity, whereas $^{\bullet}OH$ also reacts quickly without specificity and $LOO^{\bullet}$, $NO^{\bullet}{}_2$, and $ONOO^-$ have intermediate reactivity.

α- and β-tocopherol, ascorbic acid, and β-carotene). Factors that balance initiation of oxidative stress and antioxidant capacity are critical to prevent the biomolecular damage that underlies the pathogenesis of many chronic diseases caused by the overgeneration of oxidative species (e.g., ischemic injury) (Lefer and Granger, 2000; Serracino-Inglott et al., 2001).

Antioxidants are defined as substances that can potentially reduce or delay the rate of oxidation of auto-oxidizable materials. There are many naturally occurring antioxidants present in plant products that have proven efficacy for reducing the generation of free radicals that precede oxidative stress. *Echinacea* is one excellent example of a plant that contains bioactive phytochemicals with antioxidant properties. The purpose of this chapter is to detail the chemistry of antioxidant constituents present in *Echinacea* and to describe the mechanism of action.

GENERATION OF REACTIVE OXYGEN SPECIES (ROS)

Oxidations occurring both *in vitro* and *in vivo* are generally characterized by three distinct reactions, including initiation, propagation, and termination (Equation 6.1 to Equation 6.3).

Initiation:

$$LH + initiator \rightarrow L^{\bullet} \qquad (6.1)$$

$$LOOH \rightarrow LOO^{\bullet} + H$$

Propagation:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad (6.2)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Termination:

$$L^{\bullet} + L^{\bullet} \rightarrow L - L \tag{6.3}$$

$$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$$

Hall and Cuppett (1997) proposed two mechanisms of lipid oxidation whereby oxygen is required but is not necessarily exclusive. Transition metal ions and high-energy irradiation (e.g., ultraviolet light) are potential radical acceptors that generate radicals from a reaction that requires oxygen and leads to the formation of alkoxyl and peroxyl radicals. With photo-oxidation, light-sensitizing agents (such as chlorophyll) mediate the generation of a highly reactive singlet oxygen ($^1O_2$) species, which is 1500 times more reactive than its stable triplet oxygen ($^3O_2$) counterpart. This reaction in turn initiates lipid oxidation by catalyzing hydroperoxide decomposition. Antioxidants that effectively inhibit chain reactions and are characteristic of initiating free radical formation and subsequent propagation of more ROS will delay the onset of lipid oxidation, or retard the rate of chain reaction, respectively (Simic et al., 1992). Aspects of antioxidant function include mechanisms that involve free radical chain breaking, metal sequestering, and oxygen quenching (Hall and Cuppett, 1997). For example, a chain-breaking antioxidant (AH) interferes with either the initiation or propagation step and generates more stable intermediate radicals or a nonradical product (St. Angelo, 1996). Chain-breaking antioxidants are also classified as chain-breaking electron donors and chain-breaking acceptors. The chain-breaking electron donor antioxidant competes with the unsaturated fatty acid (LH) for the peroxyl radical ($LOO^{\bullet}$, Equation 6.4), thus reducing the rate of propagation (Equation 6.2). On the other hand, a chain-breaking acceptor antioxidant competes with the triplet oxygen ($^3O_2$, Equation 6.5), and as a result, reduces the propagation of free radicals by preventing the generation of $LOO^{\bullet}$ (Equation 6.2).

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \tag{6.4}$$

$$\mathrm{L}^{\bullet} + \mathrm{AH} \rightarrow \mathrm{LH} + \mathrm{A}^{\bullet} \tag{6.5}$$

Antioxidants are derived from both synthetic and natural sources. Numerous synthetic chemical agents have been used as primary antioxidants in the food industry, primarily focusing on the retention of essential nutrients (e.g., polyunsaturated fatty acids, essential amino acids) and sensory perception of quality (e.g., color pigments). Common examples of synthetic antioxidants include butylated hydroxylanisole (BHA), butylated hydroxytolulene (BHT), *tetra*-butyl-hydroquinone (TBHQ), and propyl gallate (PG). Concerns regarding the controversy over the safety of synthetic food antioxidants (Shahidi et al., 1992; Williams et al., 1999) have led to the effort to discover natural sources of materials that could be used as food antioxidants or supplements. An example of a natural antioxidant is ascorbic acid and the long-chain fatty-acid esterified form of ascorbic acid, which improves lipid solubility for the application for hydrophobic food systems (St. Angelo, 1996). Other natural sources of antioxidants reported from herbs and spices include rosemary (*Rosmarinus officinalis*) and sage (*Salvia*) extracts (Hall and Cuppett, 1997). These examples contain active phenolic compounds (e.g., carnosol, carnosic acid, rosmanol, rosmaridiphenol, and rosmarinic acid) with noted antioxidant activity. Other natural antioxidants derived from plants with potential application for health benefits include the flavonoids from *Ginkgo biloba* extracts (Yan et al., 1995; Haramaki et al., 1998), tea catechins such as epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (Weisburger, 1998; Hu and Kitts, 2001), and lignan from flaxseed (Kitts et al., 1999).

PREVENTION OF OXYGEN RADICAL–INDUCED DAMAGE BY *ECHINACEA* CONSTITUENTS

PHENOLICS

Caffeic acid derivatives represent a major group of phenolic constituents that are present in all *Echinacea* species (e.g., *E. angustifolia, E. purpurea*, and *E. pallida*) with bioactive properties that have potential uses for various medicinal purposes (Bauer and Wagner, 1991; Bauer, 1999; Bauer, 2000). Of the two major caffeic acid derivatives, cichoric acid has greater pharmacological relevance compared to echinacoside (Bauer, 2000). Structures of echinacoside and cichoric acids are shown in Figure 6.1. It is noteworthy that *E. purpurea* does not contain echinacoside (Borchers et al., 2000), which enables partial identification of *E. purpurea* from the other two species. Phenylethanoid glycosides are common constituents of the *Echinacea* species *E. pallida* and *E. angustifolia* (Bauer, 2000; Sloley et al., 2001; Hu and Kitts, 2000) and contribute to the antioxidant activity associated with free-radical scavenging properties of *Echinacea* (Zheng et al., 1993; Wang et al., 1996; Hu and Kitts, 2000).

Antioxidant activities of *Echinacea* species have been studied in different model systems using methanol extracts of *E. angustifolia*, *E. purpurea*, and *E. pallida* roots (Hu and Kitts, 2000). In both water-soluble and ethanol-soluble free-radical scavenging models, extract of *E. pallida* presented higher free radical–scavenging capacity than the other two species, which was attributed to the higher content of caffeic acid derivative found in *E. pallida*. Methanol extracts of roots of *E. angustifolia*, *E. purpurea*, and *E. pallida* were tested for suppressing peroxyl radical–induced, reconstructed, phospholipid liposome peroxidation. In this system, peroxyl radicals were generated through thermolysis of 2, 2'-azobis(2-amidinopropane) dihydrochloride (Niki, 1990), which in turn triggered the oxidation chain reaction. Delay of onset of liposome peroxidation was similar with the addition of *Echinacea* extracts and Trolox (a water-soluble analogue of α-tocopherol) (Figure 6.2).

The addition of root extracts from all three sources of *Echinacea* resulted in characteristically different, albeit greater, reduction of lipid peroxidation compared to controls (Figure 6.2). For

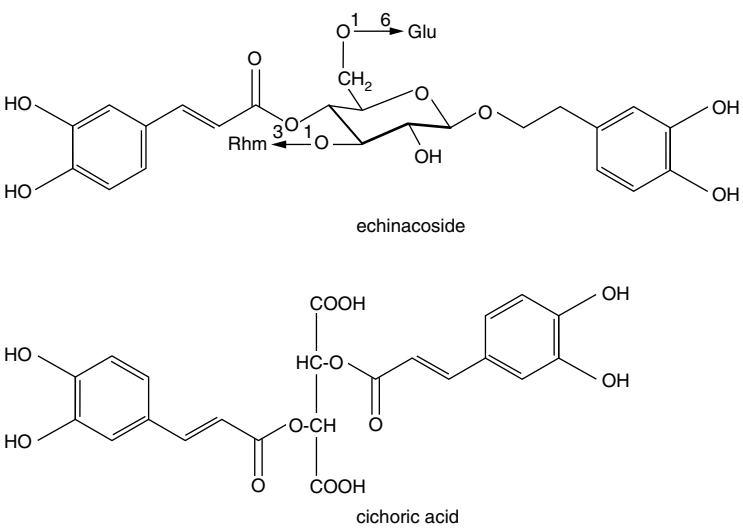

FIGURE 6.1 Structure of echinacoside and cichoric acid.

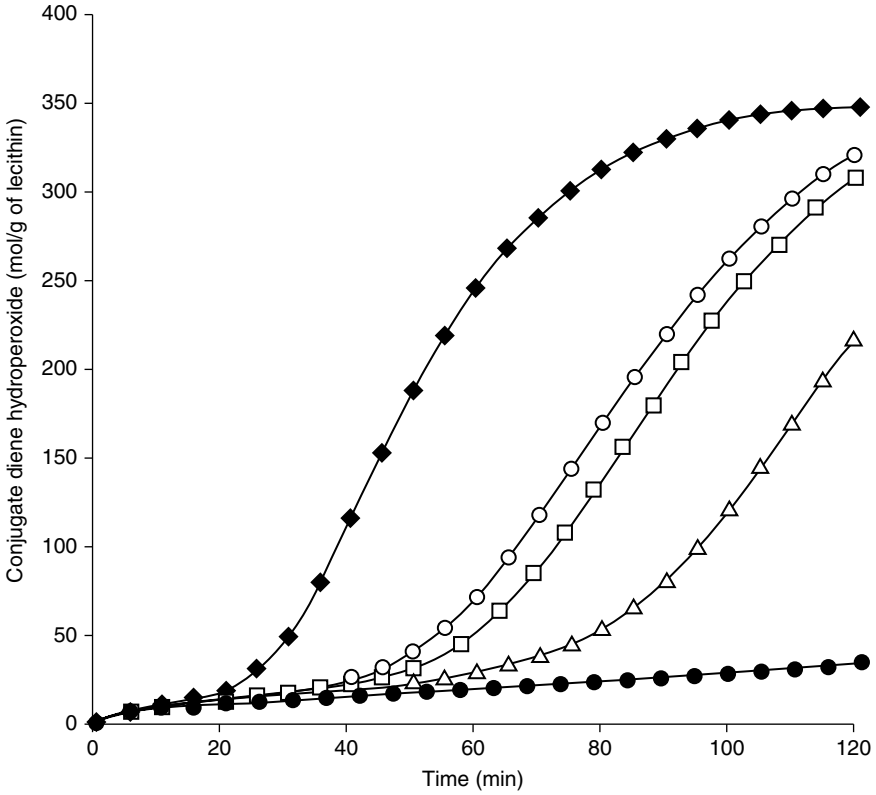

FIGURE 6.2 *Echinacea* root extracts (5µg/ml) protect liposome from peroxyl radical induced oxidation at 37°C. ◆ = control, □ = *E. angustifolia*, △ = *E. pallida*, ○ = *E. purpurea*, ● = 2.5µg/ml of Trolox.

example, the pattern of protection of phospholipid-rich constructed liposome against peroxyl radical–induced oxidation is characterized by a prolonged duration of the initial phase and suppression of the rate of propagation (Equation 6.1 and Equation 6.2, respectively). The reduced rate of propagation of oxidation reaction is seen by the flatter slope for the propagation curve, thus indicating characteristics of free radical chain-breaking antioxidant activity.

Cichoric acid is present in both flower heads (1.2% to 3.1% w/w) and roots (0.6% to 2.1%, w/w) of medicinal varieties of *Echinacea*, and its concentration depends on the species sources of *Echinacea* as well as the season of harvest (Bauer, 2000). Facino et al. (1995) showed that echinacoside exhibited a stronger protection against free radical–induced native collagen degradation than other caffeic acid derivatives, such as cichoric acid, caffeic acid, and chlorogenic acid. In this *in vitro* model system, hydroxyl and superoxide anion radicals were generated by incubating the combination of xanthine–xanthine oxidase and Fe^{2+} with EDTA (Equation 6.6 and Equation 6.7).

$$xanthine + O_2 + H_2O \xrightarrow{xanthineoxidase} uricacid + H_2O_2 + O^{\bullet-}_2 \tag{6.6}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + HO^{\bullet} \tag{6.7}$$

It was clear that the caffeic acid derivatives did not directly interfere with xanthine oxidase activity, and thus displayed a notable degree of antioxidant capacity by directly scavenging free radicals. As a result of this indication, a topical application was proposed for use of *Echinacea* extract to prevent or treat photo-damaged skin from ultraviolet irradiation (Black et al., 1997). Some studies have not discriminated a plant species–specific difference in affinity to quench hydroxyl radical (Hu and Kitts, 2000), while other reports have shown that an *E. purpurea* root extract possessed relatively higher hydroxyl radical scavenging capacity compared to *E. angustifolia* and *E. pallida* (Arnao et al., 1996; Sloley et al., 2001). There is agreement that the relative content of cichoric acid in *E. purpurea* root is higher than the other two *Echinacea* species (Hu and Kitts, 2000; Sloley et al., 2001). However, the reason for the different results concerning relative capacity to scavenge for hydroxyl radicals may not be solely attributed to the chemical composition but also to the different methods used to test for free radical scavenging activity.

There is further evidence to show that pure caffeic acid derivatives have direct free radical–scavenging activity, as evidenced by the quenching capacity demonstrated toward the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH). In this test, decolorization of the reaction mixture is the indication of free radical–scavenging capacity for the antioxidant agent (Blois, 1958). Xiong et al. (1996) demonstrated that echinacoside from *Cistanche deserticola* strongly inhibited DPPH radical and superoxide anion radical, the latter generated from xanthine–xanthine oxidase reaction (Equation 6.6). Moreover, echinacoside effectively reduced lipid peroxidation in rat liver microsome induced by both enzymatic and nonenzymatic procedures. Echinacoside isolated from Pedicularis has also been reported to protect against oxidative hemolysis *in vitro* (Li et al., 1993) and autoxidation of linoleic acid in acetyl trimethylammonium bromide (CTAB) micelles (Zheng et al., 1993). The antioxidant activity of echinacoside was attributed to both the number and the position of phenolic hydroxyl groups that substitute the molecular moieties (Li et al., 1993; Zheng et al., 1993; Wang et al., 1996).

The concentration of free phenolic acids present in aerial parts of different *Echinacea* species has also been determined using high-performance liquid chromatography (HPLC) (Glowniak et al., 1996). Total phenolic acids vary from 73 μg/g dry weight (e.g., *E. umbellate*) to 138 μg/g dry weight (*E. commutata*). Leaf extract derived from *E. angustifolia, E. purpurea*, and *E. pallida* exhibit lower antioxidant activities compared to respective root extracts (Sloley et al., 2001). Pure cichoric acid derived from *E. purpurea* root has been shown to provide more than three times greater hydroxyl radical–scavenging activity than ascorbic acid when compared on an equal molar basis. Leaf extract presented a relatively greater affinity than respective root extracts at preventing Fe^{2+}-induced lipid oxidation in a catecholaminergic neuroblastoma SH-SY5Y cell line. This result occurred despite the fact that there was no difference between various *Echinacea* species.

In addition to the antioxidant activities of echinacoside and cichoric acid reported in various *Echinacea* species, other phytochemical constituents, especially chlorogenic and isochlorogenic acids, have been identified in both leaf and root of *E. pallida* and *E. angustifolia* (Bauer and Wagner, 1991) and possess antioxidant activity. The antioxidant affinity of chlorogenic acid has been well studied using the 2, 2'-azinobis(3-ethylenzothiazoline-6-sulfonic acid) radical (e.g., $ABTS^{\bullet+}$) model (Miller, 1998). Both chlorogenic and caffeic acids have been shown to exhibit antioxidant equivalents 1.24 and 1.26 times of Trolox, respectively, while quercetin is almost four times greater. Compared to echinacoside and cichoric acid, both chlorogenic and isochlorogenic acids represent relatively minor constituents of *Echinacea*. The standard procedure for preparing *Echinacea* is therefore based mostly on the presence of echinacoside and cichoric acid, rather than chlorogenic acid (Bauer and Wagner, 1991). Thus, the relative significance of antioxidant activity of chlorogenic acid in various *Echinacea* preparations is not fully appreciated. It is of interest that in other products such as apple juice, the antioxidant activity of chlorogenic acid can contribute as much as 41% of all antioxidant activity in the packaged apple juice (Miller, 1998).

Phagocytosis is part of nonspecific immune responses to infection. During phagocytosis, an oxygen consumption burst generates superoxide ions and hydrogen peroxide, while a more reactive, oxidative hydroxyl radical is also generated through the Fenton reaction mechanism (Equation 6.8).

$$H_2O_2 + Fe^{2+}\left(Cu^{+}\right) \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+}\left(Cu^{2+}\right) \tag{6.8}$$

Phagocytosis of yeast by human granulocytes *in vitro* is associated with the burst of oxygen radicals (Follin and Dahlgren, 1992), resulting in the enhancement of luminol-mediated chemiluminescence. Extracts from both *E. angustifolia* and *E. purpurea* stimulated the chemiluminescence in this assay (Borchers et al., 2000). However, results obtained from a similar assay were inconsistent (Gaisbauer et al., 1990) and may again be attributed to the different experimental model used to evaluate the bioactive response (Borchers et al., 2000).

Flavonoids

In addition to chlorogenic and caffeic acids, other compounds present in *Echinacea* that contribute to the antioxidant activity are the flavonoids. Flavonoids are a large group of naturally occurring compounds that have a characteristic C6-C3-C6 skeleton, with different sites that possess varying degrees of hydroxylation and/or glycosidation (Van Acker et al., 1995; Rice-Evans et al., 1996; Arora et al., 1998). These chemical moieties determine the relative antioxidant activity of the compound. For example, the *ortho*-di-hydroxyl group located on the B-ring is a critical structure required for free radical scavenging activity (Arora et al., 1998; Rice-Evans et al., 1996). Flavonoid concentrations in *Echinacea* are relatively low. In *Echinacea* leaf, common flavonoids found include luteolin, kaempferol, quercetin, quercetin-7-galactoside, luteolin-7-glucoside, kaempferol-3-glucoside, quercetin-3-arabinoside, quercetin-3-galactoside, quercetin-3-xyloside, quercetin-3-glucoside, kaempferol-3-rutinoside, rutoside, and isorahmnetin-3-rutinoside (Figure 6.3). Rutoside is a major flavonoid present in the leaves of *E. angustifolia, E. pallida*, and *E. purpurea* (Bauer and Wagner, 1991).

A systematic study of the antioxidant activity of the mixture of *Echinacea* flavonoids is not available; however, individual flavonoids have been characterized for antioxidant activity using a number of different model systems. Antioxidant activity of flavonoid has been demonstrated in the low-density lipoprotein (LDL) forced peroxidation model using cupric ion (Vinson et al., 1995; Morel et al., 1998; Hu and Kitts, 2001). One factor that is critical for the catalytic peroxidation of LDL by Cu^{2+} is the likely presence of small amounts of peroxide that converts Cu^{2+} to the active Cu^{+} form (Esterbauer et al., 1992). The catalytic breakdown of lipid hydroperoxide derived from LDL phospholipid surface or the cholesteryl ester core results in modification of the phospholipid and cholesteryl ester and propagation of free radicals. These reactions in turn modify the lysine residues of apo-B on the LDL particle. The oxidation of LDL (ox-LDL) produces a change in surface charge that can be evaluated by observing the migration behavior on agarose gel electrophoresis, or alternatively, increased response in fluorescence spectrophotometry. If we employ this model using the *in vitro* forced peroxidation of human LDL (100 µg protein/mL) by 10 µM Cu^{2+}, we show here that equal antioxidant activity of both 100-µM luteolin and quercetin produces similar protection against LDL oxidation, as measured by reduced migration of ox-LDL on agarose gel electrophoresis (Table 6.2). Kaempferol, which is characterized by the absence of a B-catechol group, produces a markedly lower protection against LDL oxidation. The presence of multiple hydroxyl groups on the flavonoid structure brings forth the antioxidant activity by also providing metal ion chelation properties. This property is related to the fact that the presence of free Fe^{2+} and Cu^{+} metal ions will lead to the formation of highly reactive hydroxyl radicals through the Fenton reaction (Equation 6.7 and Equation 6.8). Quercetin is a good example of a flavonoid forming a ligand with metal ions (Takahama, 1985), leading to a characteristic shift of absorption spectra

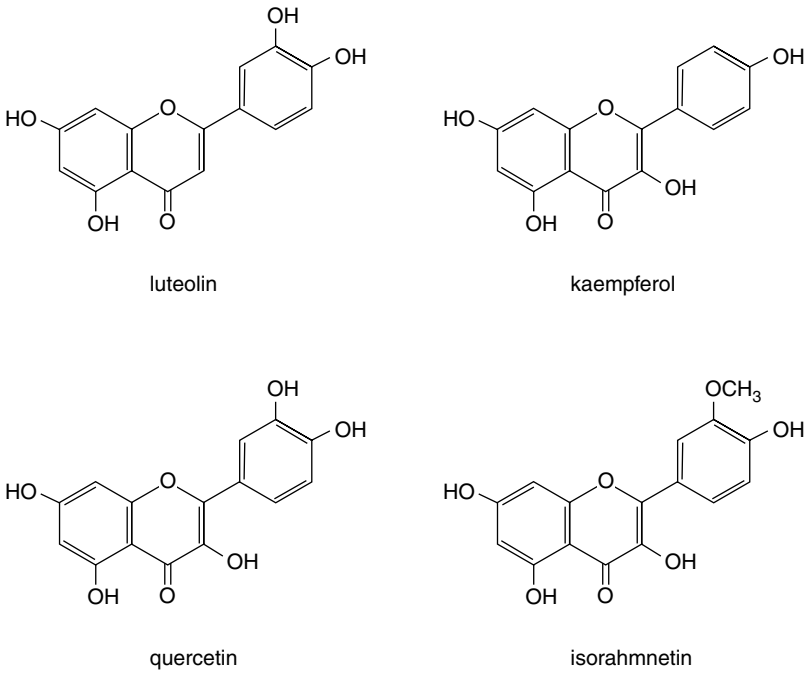

FIGURE 6.3 Structure of flavonoid aglycon found in *Echinacea* leaves.

TABLE 6.2
Flavonoid Found in *Echinacea* Leaf and Corresponding Affinity to Prevent LDL Oxidation[a]

Flavonoid	Inhibition Percentage at 100 μM
Luteolin	71.1±1.2
Kaempferol	77.8±2.1
Quercetin	73.5±5.4
Luteolin-7-glucoside	78.8±1.9
Rutin	55.3±7.7

[a] Expressed as percent inhibition of cupric ion–induced LDL. Values represent the percent decrease in mobility of human LDL migration on agarose gel electrophoresis. (From CU and DDK, unpublished results, 1998.)

(Afanas'ev et al., 1989; Wu et al., 1995). Potential pro-oxidant activity that will occur between plant phenolics and free transition metal ions must also be considered when evaluating the antioxidant activity of flavonoids. The characteristic redox potential of flavonoids acts to reduce the transition metal ion to a lower valence form that is favorable for the Fenton reaction, and in turn accelerates a pro-oxidant reaction. This property has been reported for both a complex extract (Hu et al., 2000) and a purified catechin, such as epigallocatechin gallate (Hu and Kitts, 2001), where free transition metal ions were present.

Other studies have evaluated the antioxidant activity of these flavonoids using other models, such as the methyl linoleate micelle. It is clear from various studies that flavonoids represent moderate chain-breaking agents that scavenge lipid alkoxyl radicals and peroxyl radicals by acting

as a chain-breaking electron donor (Rice-Evans, 1995; Roginsky et al., 1996). The rate constant of flavonoids reacting with peroxyl radical has been estimated to be $1 \times 10^7 M^{-1}s^{-1}$ (Bors et al., 1994; Belyakov et al., 1995). Specifically, the B-ring catechol structure along with the 2, 3-double bond and 3, 5-hydroxyl groups on the flavonoid backbone is closely related to antioxidant activity (Roginsky et al., 1996). These chemical moieties and structural components of flavonoids furthermore determine the extent of partition between the hydrophilic and hydrophobic phase in a heterogeneous system (Foti et al., 1996).

Another model used to assess antioxidant activity of plant phenolics common in *Echinacea* is the phospholipid bilayer. Flavonoids such as quercetin are more effective than α-tocopherol, which is mainly responsible for chain-breaking activity against lipid peroxidation products that are exposed to water-soluble peroxyl radicals (Terao et al., 1994). Not withstanding this, however, is the noteworthy finding that the reducing capacity of flavonoids contributes also to indirect properties of antioxidant activity by regenerating α-tocopherol (Mukai et al., 1996). This characteristic explains the unique disappearance rate of α-tocopherol and quercetin if the chain initiation occurs within the membrane (Terao and Piskula, 1998). This model has also shown that quercetin is not as effective as α-tocopherol at scavenging chain-propagating, lipid peroxyl radicals in a hydrophilic phase. This observation is largely due to the localization of quercetin in the aqueous phase, and thus lower affinity to interact with lipid peroxyl radicals that are residing with α-tocopherol in hydrophilic zones of the suspension.

Further evidence of characteristic bioactive properties of different flavonoids can be seen in cytotoxicity studies assessed *in vitro* with various cell lines. In the example shown below, luteolin exhibited the strongest cytotoxicity against caco-2 cells compared to luteolin-7-glucoside and other flavonoids (Figure 6.4). It is noteworthy that a pattern for relative antioxidant activity and cytotoxicity is present for the various flavonoids.

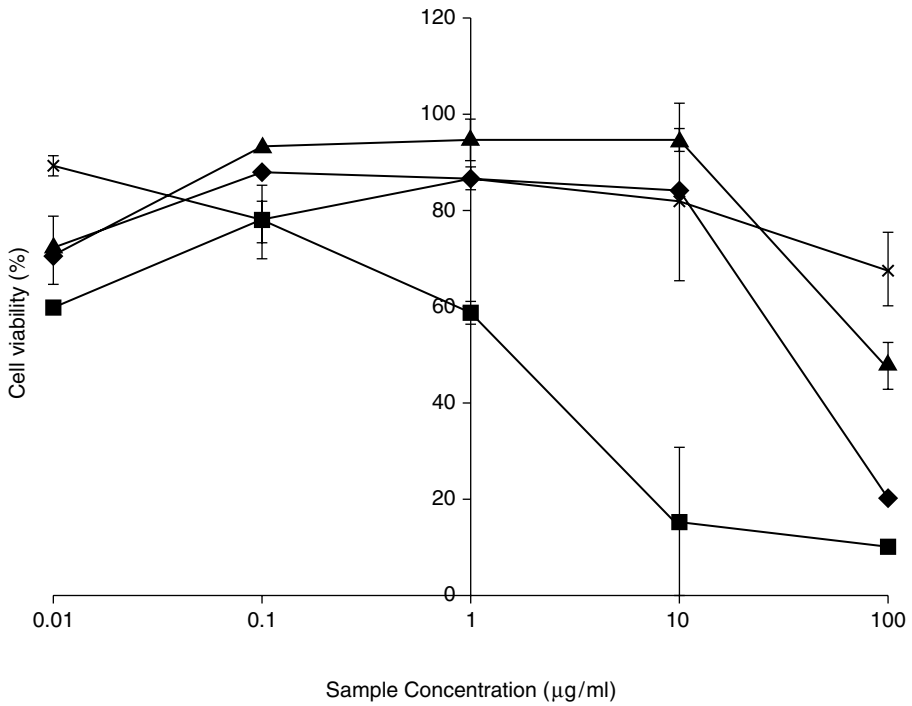

FIGURE 6.4 Cell viability of Caco-2 cell incubated with different flavonoid found in *Echinacea*. ♦ = luteolin-7-glucoside, ■ = luteolin, ▲ = quercetin, x = rutin. (CU and DDK, 1999.)

Anthocyanins

Anthocyanins are one of many distinct groups in the flavonoid family that have been identified as having various health benefits. Flavonoids account for a large proportion of phenolic phytochemicals in the human diet from such sources as tea, vegetables, and fruits, and derivatives (Cook and Samman, 1996). The consumption of particular flavonoids, such as catechin, typically varies by age and gender (Arts et al., 2001) and is partially explained by dietary habits. Epidemiological studies have shown a negative relationship between chronic exposure to flavonoids and incidence of coronary heart disease and ischemic heart disease (Hertog et al., 1997a, 1997b). For example, moderate wine consumption has been linked to the antioxidant properties of anthocyanins and reduced risk of cardiovascular disease (Cao et al., 1998; Wollin and Jones, 2001). In a 4-week clinical trial, human subjects with regular tea consumption exhibited a significantly prolonged LDL oxidation *ex vivo* compared to the placebo (Ishikawa et al., 1997). Anthocyanin has also been detected in both human and animal blood after consumption, thereby indicating the absorption and possible metabolism of these compounds (Tsuda et al., 1999; Cao et al., 2001). Grape juice, a good source of both anthocyanin and proanthocyanin, has also been shown to extend the lag phase for human LDL oxidation and increase flow-mediated vasodilation compared to controls (Stein et al., 1999).

Anthocyanins provide in large part the plant pigments found in the *Echinacea* flower (Cheminta et al., 1989). The principal anthocyanins present in the *Echinacea* flower are cyanidin-3-O-β-glucopyanoside and cyanidin-3-O-6-malonyl-β-D-glucopyranoside. Anthocyanins are also abundant in berries, fruits, and grapes (Cliford, 2000). The antioxidant property of cyaniding-3-glucoside has been demonstrated in various test model systems (Tsuda et al., 1994). Using the conditions outlined in the legend in Figure 6.5, cyanindin-3-glucoside derived from a blackberry source suppresses DNA damage that is mediated by peroxyl radicals. The potential benefit of anthocyanin from *Echinacea* remains to be determined due to the fact that flowers are less used in this herbal preparation.

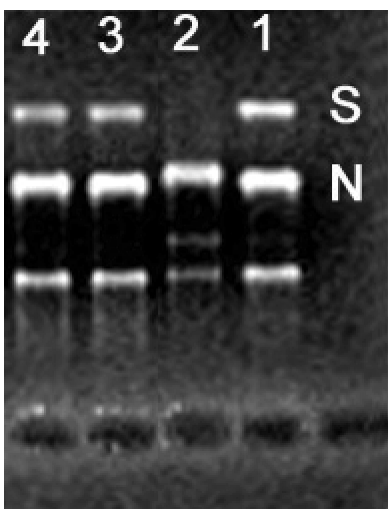

FIGURE 6.5 Effect of freeze-dried and frozen blackberry extracts on preventing peroxyl radical–induced supercoiled DNA from nicking. S = supercoiled DNA; N = nicked DNA strand; lane 1 = DNA + PBS; lane 2 = DNA + peroxyl radical + PBS; lane 3 = DNA + peroxyl radical + 0.05 mg/mL freeze-dried blackberry extract; lane 4 = DNA + peroxyl radical + 0.05 mg/mL frozen blackberry extract.

EFFECTS OF *ECHINACEA* EXTRACTS ON NITROGEN RADICALS

Nitric oxide (NO) with an unpaired electron reacts as a free radical. The production of nitric oxide in mammalian cells by the oxidation of L-arginine by nitric oxide synthase (NOS) includes both constitutional (cNOS) and inducible NOS (iNOS) forms (Nathan and Hibbs, 1991). The NO level in a normal physiological condition is low until the expression of iNOS occurs, which leads to increased amounts of NO production. The level of iNOS expression is determined partially by the rate of transcription, which is dependent on NF-κB activation (Xie et al., 1994). Activation of cells by appropriate stimuli results in the phosphorylation, uniquination, and degradation of IκB, which liberates NF-κB to translocate into nuclei and interact with a κB motif on the promoter of target genes such as iNOS (Han et al., 2001). Oxidative stress was found to be partially responsible for protein phosphorylation (Ushio-Fukai et al., 1998), NF-κB activation (Schreck et al., 1991; Meyer et al., 1993), and oxidative stress gene expression (Lee and Corry, 1998). Pro-inflammatory agents such as bacterial lipopolysaccharide (LPS) or IL-1β, TNF, and IFN-γ will stimulate the expression of iNOS. In mouse macrophages, LPS-induced expression of iNOS depends on the activation of NF-κB heterodimer p50/c-rel and p50/Rel A (Xie et al., 1994), and expression of iNOS leads to the production of massive amounts of NO. The reaction between NO and superoxide anion results in the generation of a highly reactive nitrogen species peroxynitrite ($ONOO^-$), bringing the NO into the category of a pro-oxidant (Violi et al., 1999). The reason for high oxidative activity of $ONOO^-$ lies with the weak strength of the O-O bond and spontaneous decomposition to form hydroxyl radical and nitrogen dioxide (Koppenol et al., 1992).

Recently, echinacoside isolated from *Cistranche deserticola* stem was found to suppress the generation of nitric oxide in J774.1 macrophage cells cultured with lipopolysaccharide and mouse perifoneal macrophage stimulated with LPS and IFN-g (Xiong et al., 2000). No inhibition on iNOS mRNA expression was found; consequently, neither were the iNOS protein found nor the iNOS activity in lipopolysaccharide-stimulated macrophage enhanced. Therefore, the inhibition of the generation of nitric oxide was attributed to the direct scavenging of nitric oxide. Evidence of such direct scavenging was observed in a system with PAPA NONOate, which generates nitric oxide radical by spontaneous dissociation (Xiong et al., 2000). Therefore, the authors concluded that phenyethanoids, including echinacoside, were unlikely to inhibit NF-κB activation; a reaction which differs from the antioxidant (-)-epigallocatechin-3-gallate from green tea, known to suppress nitric oxide in a manner of inhibition of NF-κB activation (Lin and Lin 1997).

Crude extracts of *Echinacea* (e.g., *E. purpurea*) that contain cichoric acid, polysaccharide, and alkylamide have been reported to reduce the nitric oxide release from rat alveolar macrophages that were stimulated with LPS. Of the three chemical identities, alkylamide was the most effective, and also increased the production of TNF-a in alveolar macrophage cells in a concentration-dependent manner. These results support the *in vivo* evidence that alkylamide from *Echinacea* extract can be an effective nonspecific immunomodulatory agent (Goel et al., 2002). *Echinacea* extracts chemically standardized to phenolic acid or echinacoside content and fresh pressed juice preparations were found to display antiinflammatory and antioxidant properties in varying degrees, as shown in the suppression of prostaglandin E_2 in mouse macrophage RAW264.7 cell treated with IFN-a (Rininger et al., 2000).

Similar to the evidence shown with reactive oxygen species, flavonoids play an important role in suppressing nitric oxide production. Kim et al. (1999) found that the structural features in favor of strong activity to reduce nitric oxide include the C-2, 3-double bond and 5, 7-dihydroxyl groups in the A-ring. The 3-hydroxyl moiety in the C-ring will actually reduce the activity. For example, luteolin reduces the iNOS enzyme expression in LPS-activated RAW264.7 cell in a concentration-dependent manner without inhibition of enzyme activity itself. In terms of transcription, flavonoid-rich *Ginkgo biloba* (EGb 761) and its major flavonoid, quercetin, were found to inhibit p38 mitogen-activated protein kinase (MAPKs) activity, which is necessary for the iNOS expression in LPS-

stimulated RAW264.7 macrophages. However, quercetin had no effect on LPS-induced activation of NF-κB (Wadsworth and Koop, 2001).

EFFECTS OF *ECHINACEA* EXTRACTS ON OXIDATIVE ENZYMES

Polyphenol oxidase (PPO, EC 1.14.18.1) catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. These reactions lead to the generation of brown color, termed enzymatic browning. This browning reaction occurs in vegetables, fruits, and herbs during postharvest handling and results in a loss of quality that adversely affects acceptability by consumers (Martinez and Whitaker, 1995). Wolfgang et al. (2000) purified PPO from *E. purpurea*, which has a high affinity for caffeic, cichoric, and rosmarinic acids that represent enzyme substrates. It was of interest to note that PPO from *E. purpurea* also possesses diphenolase activity in addition to monophenolase activity. As a matter of fact, Nüsslein et al. (2000) found the existence of polyphenol peroxidase catalyzed cichoric acid degradation in *E. purpurea* preparation, suggesting the necessity of increasing ethanol concentration in order to inhibit enzyme activity in processing. Similarly, Kim et al. (2000a) demonstrated the decline of caffeic acid derivatives in *E. purpurea* flower during drying processing. Taking these findings together, it is clear that caffeic acid derivatives, such as cichoric acid from *Echinacea*, are sensitive to environmental and herbal processing and handling, therefore extra measures are required for the preparation of this herb. For example, use of low temperature, elevated ethanol concentration, and storage in a low-humidity environment reduces the loss of both alkamide and cichoric acid from *E. purpurea* (Stuart and Wills, 2000; Wills and Stuart, 2000). Application of metal-chelating agents to deactivate cupric ions in the active site of PPO (Wolfgang et al., 2000) was also found to be useful in order to maximize the retention of cichoric acid in the preparation.

An *in vitro* screening test widely used to determine the antiinflammatory activity of *Echinacea* is the inhibition of cyclooxygenase and 5-lipoxygenase (Celotti and Laufer, 2001; Bernrezzouk et al., 2001). These two enzymes are central to the pathway producing thromboxanes, prostaglandins, and leukotrienes (Borchers et al., 2000). Non-heme iron-centered lipooxygenase exists in both animal and plant tissues. The enzyme catalyzes the oxidation of polyunsaturated fatty acid with conjugated diene substructure, such as linoleic acid and arachidonic acid. One mechanism of lipoxygenase is the oxidation and deprotonation of diene to generate a pentodienyl radical. When oxygen is subsequently added to form a fatty acid peroxyl radical, the ferrous ion is regenerated back to a ferric form (de Groot et al., 1975). Alternatively, the diene substrate can be deprotonated and coordinated with ferric ion to form a σ-organometallic complex, where di-oxygen, when inserted to break down the Fe-C bond, resulted in fatty acid hydroperoxide and the regeneration of enzymes (Corey and Nagata, 1987). Both cyclooxygenase and 5-lipoxygenase are critical for the arachidonic acid metabolism and associated with the formation of leukotrienes and prostaglandins. The alkamide fractions from both *E. purpurea* and *E. angustifolia* inhibit 5-lipoxygenase activity (Wagner et al., 1989). Specifically, eight alkamides isolated from *E. angustifolia* have shown different inhibitory activities on both enzymes *in vitro.* Specifically, pentadeca-2E, 9Z-diene-12, 14-diynoic acid isobutylamide, and dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide exhibit the highest inhibitory activities against cyclooxygenase and lipoxygenase activities, respectively. The mechanism underlying this inhibition has been suggested to involve the enzymatic competition between structurally similar alkamides and arachidonic acid in the reaction. Moreover, possible redox-inhibitory properties or radical scavenging capacities may also be involved (Müller-Jakic et al., 1994). Significant consideration should also be given to dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide activity, since both account for the predominant alkamide in the lipophilic fraction from *E. angustifolia* (root), *E. purpurea*, and *E. pallida* (Bauer and Remiger, 1989). *E. tennesseensis* contains only low quantities of dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide (Bauer et al., 1990). Taking the antioxygenase activity into account, it is necessary

to emphasize the importance of postharvest procedures in order to maximize the retention of biologically relevant alkamides. For example, Kim et al. (2000b) observed that freeze-dried *Echinacea* root resulted in the best retention of dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide and other individual alkamides, compared with conventional air drying. Perry et al. (1997) analyzed the distribution of alkamide in different parts of *E. purpurea*, and noted that dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide was most abundant in the vegetative stem, albeit this part accounts for only 2% of the whole plant.

FINAL COMMENTS

The literature reviewed above describes the potent antioxidant and free radical scavenging activities of *Echinacea* extracts and individual components, albeit many of these studies were conducted *in vitro*. It is not clear if these same components from *Echinacea* possess similar bioactivity *in vivo,* and whether or not the antioxidant properties claimed potentiate the reported health benefits of *Echinacea*, such as antiinflammation and general cold relief. Pharmacokinetic data are currently unavailable for the major antioxidant components present from *Echinacea*. It is important that this information be obtained in order to understand how antioxidant components derived from *Echinacea* work *in vivo* to trigger other related protein expressions. For example, isoflavones genistein and daidzein significantly increased the expression of antioxidant protein metallothionein in human intestinal Caco-2 cells (Kameoka et al., 1999). This effect was decreased by the treatment of quercetin (Kuo et al., 1998). In fact, catalase and Cu/Zn superoxide dismutase in Caco-2 cells were not affected by exposure to 100 μM of flavonoids, thereby suggesting that the effects of flavonoids on the antioxidant protein expression are possibly related to the specific structure of the compound. Similar studies are required with specific *Echinacea* phytochemicals.

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Section IV

Analytical Evaluation in Genus Echinacea

7 Analytical Profiles of *Echinacea* Species

Piergiorgio Pietta, Pierluigi Mauri, and Nicola Fuzzati

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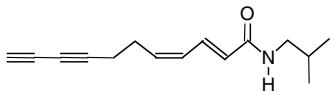
INTRODUCTION

Preparations of aerial parts and roots from three *Echinacea* species (e.g., *E. angustifolia* DC., roots, *E. pallida* Nutt., roots and *E. purpurea* Moench, roots and tops) are generally employed for treatment of cold, flu, and chronic respiratory infections (Grimm and Muller, 1999). *Echinacea* species contain a variety of components that may contribute to the nonspecific enhancement of the immune system (Bauer et al., 1998) and to antiinflammatory properties (Müller-Jakic et al., 1994). It is accepted that these activities depend on the combined action of the following categories of compounds: polar caffeoyl conjugates and polysaccharides and lipophilic alkamides and polyacetylenes. In particular, caffeic acid derivatives and alkamides have been proven to contribute considerably to the biological properties of *Echinacea* species. Among caffeic acid derivatives, cichoric acid (dicaffeoyl tartaric acid) is known to have *in vitro* and *in vivo* immunomodulatory activity. Moreover, it inhibits a key enzyme (hyaluronidase) involved in bacterial infection (Bauer and Wagner, 199l). Another caffeoyl derivative, echinacoside, has weak antibacterial and antiviral properties, although it does not seem to have any immunomodulatory relevance (Bauer, 1999). The alkamide fraction, which consists mainly of isobutyl amides of straight fatty acids with double or triple bonds (Figure 7.1), also strongly stimulates the phagocytic activity of granulocytes and possesses light-mediated toxicity to various *Candida* spp. (Binn et al., 2000; Goel et al., 2002). In addition, several alkamides have been shown to exert antiinflammatory activity by inhibiting the synthesis of prostaglandins and leukotrienes (Müller-Jakic et al., 1994).

Because of their proven pharmacological properties, both caffeoyl conjugates and alkamides may be suitable phytochemical markers for the *Echinacea* species. This chapter aims to review the methods currently applied for the detection of major phenolic compounds and alkamides, including high performance liquid chromatography (HPLC), micellar electrokinetic chromatography (MEKC) and mass spectrometry (MS).



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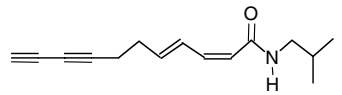

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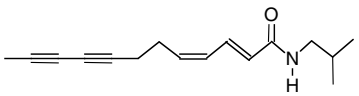

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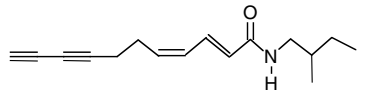

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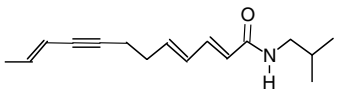

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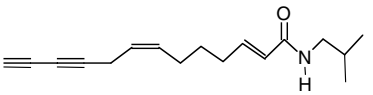

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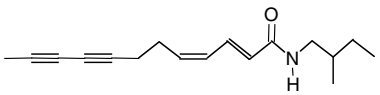

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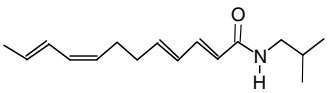

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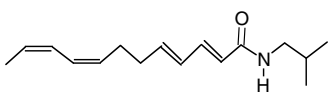

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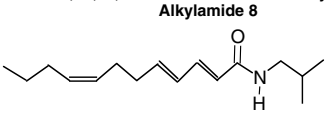

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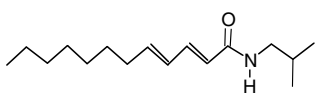

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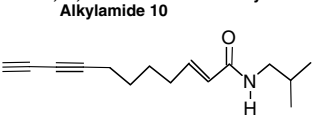

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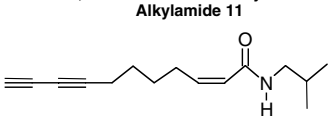

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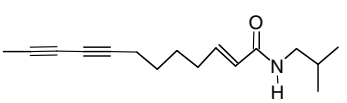

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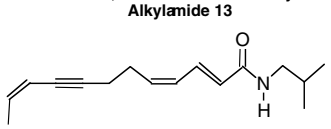

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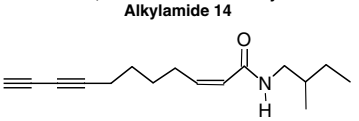

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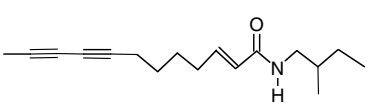

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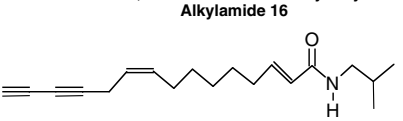

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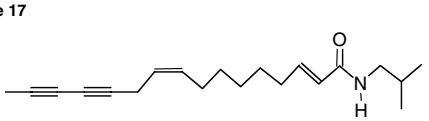

hexadeca-2*E*,9*Z*-diene-12,14-dienoic acid isobutylamide
Alkylamide 19

FIGURE 7.1 Alkamides.

Phytochemical Pattern of *Echinacea* Species

Each of the three *Echinacea* species commonly used has a distinct phytochemical profile, described briefly in this section.

Caffeic Acid Derivatives

Echinacea angustifolia Roots

Echinacoside is the main polar constituent in *Echinacea angustifolia* roots, where it is present at a concentration of 0.3% to 1.7% (Schenk and Franke, 1996). Echinacoside also occurs in *E. pallida* roots; thus it cannot be used to discriminate these two species. However, they can be easily differentiated by the presence of cynarin (1.3-dicaffeoyl-quinic acid), which is typical of *E. angustifolia* roots.

Echinacea pallida Roots

Echinacoside has been found in *E. pallida* roots at levels comparable to those measured in *E. angustifolia* roots. However, the presence (although at lower levels) in *E. pallida* roots of another phenolic, 6-O-caffeoylechinacoside (Cheminat et al., 1988) permits identification of this species.

Echinacea purpurea roots and aerial parts

The roots of *E. purpurea* are characterized by the presence of cichoric acid (2R,3R-dicaffeoyl-tartaric acid) and caftaric acid (monocaffeoyl-tartaric acid). The content of cichoric acid is in the range of 0.6% to 2.1% in fresh plant material, but decreases during manufacturing. Indeed, cichoric acid is sensitive to enzymatic degradation, and this may explain the differences in content reported for *E. purpurea* preparations. Cichoric acid is quite abundant also in the flowers of *E. purpurea*, but much less has been found in leaves and stems (Bauer, 1997).

Alkamides

Echinacea angustifolia roots

In total, 14 alkamides have been identified in *E. angustifolia* roots. They are mainly undeca- and dodecanoic acid derivatives, and differ in the number and configuration of the double bonds. The major representatives are 2-monoene-8,10-dynoic acid isobutylamides, and the main constituents (0.01% to 0.15%) are the isomeric dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (8/9). However, this pair occurs also in *E. purpurea* roots (Bauer and Reminger, 1989) and, consequently, it cannot be used to discriminate between the two species. Nevertheless, since a number of other alkamides, namely the alkamides 12 to 19, are typical of *E. angustifolia* roots, identity as well as possible adulteration with *Parthenium integrifolium* roots can be ascertained.

A similar pattern of alkamides has been described for *E. angustifolia* aerial parts, but the content of 8/9 is lower (0.001% to 0.03%).

Echinacea purpurea roots

In contrast to *E. angustifolia*, most of the 11 alkamides identified in *E. purpurea* roots have a 2,4-diene moiety, representing the main representative alkamides 8/9 and the less abundant 1 to 5 and 10. These last alkamides have UV spectra different from those of alkamides (12 to 19)

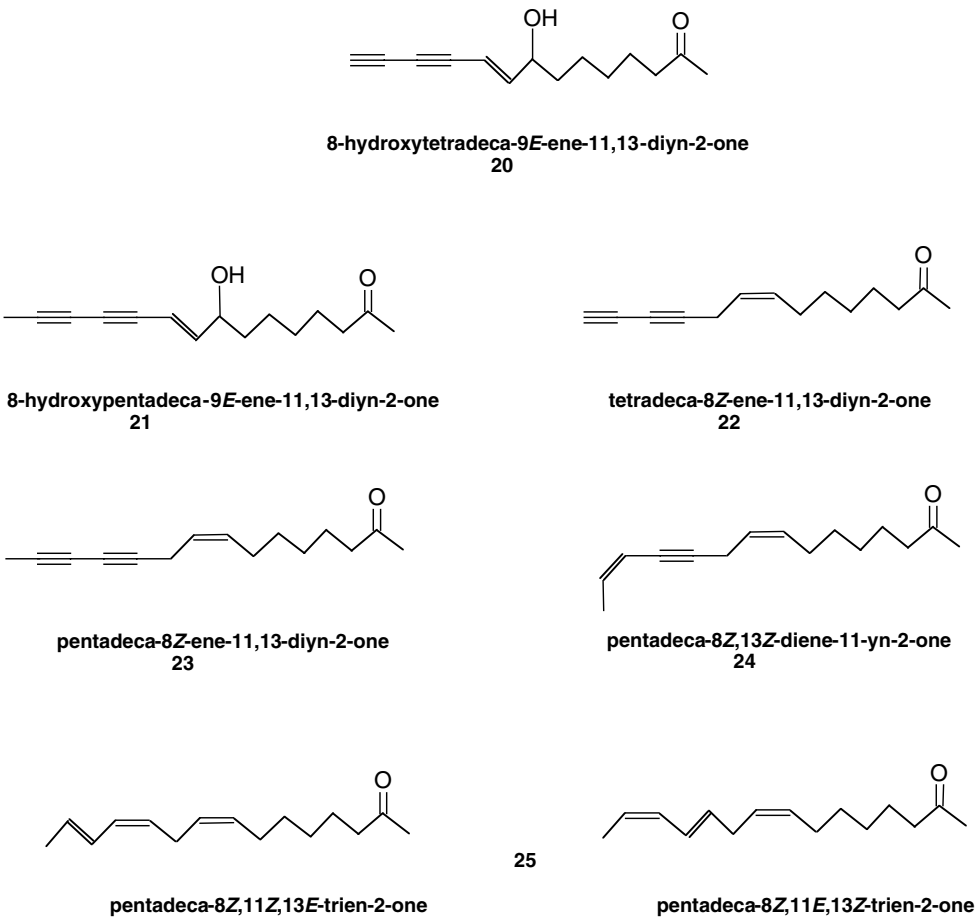

FIGURE 7.2 Ketoalkenes and ketoalkynes.

occurring in *E. angustifolia,* and HPLC coupled to diode array detection allows identification of each species.

Echinacea pallida roots

Echinacea pallida roots do not contain alkamides, but rather a number of ketoalkenes and ketoalkynes (Bauer and Reminger, 1989; Lienert et al., 1998) (Figure 7.2). The most relevant is pentadeca-8Z,13Z-dien-11-yn-2-one (24) followed by 8-hydroxyl-tetradeca-9E-ene-11,13-diyn-2-one (20), tetradeca-8Z-ene-11,13-diyn-2-one (22), pentadeca-8Z-ene-11,13-diyn-2-one and pentadeca-8Z,11E,13E/Z-trien-2-one (25).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Phenolic Compounds

Reversed phase HPLC for the analysis of the main phenolic compounds in *Echinacea* species was first applied by Bauer et al. (1988). Figure 7.3 shows the HPLC profile of methanolic extracts from the three species. This approach has been followed by other groups who used different extraction

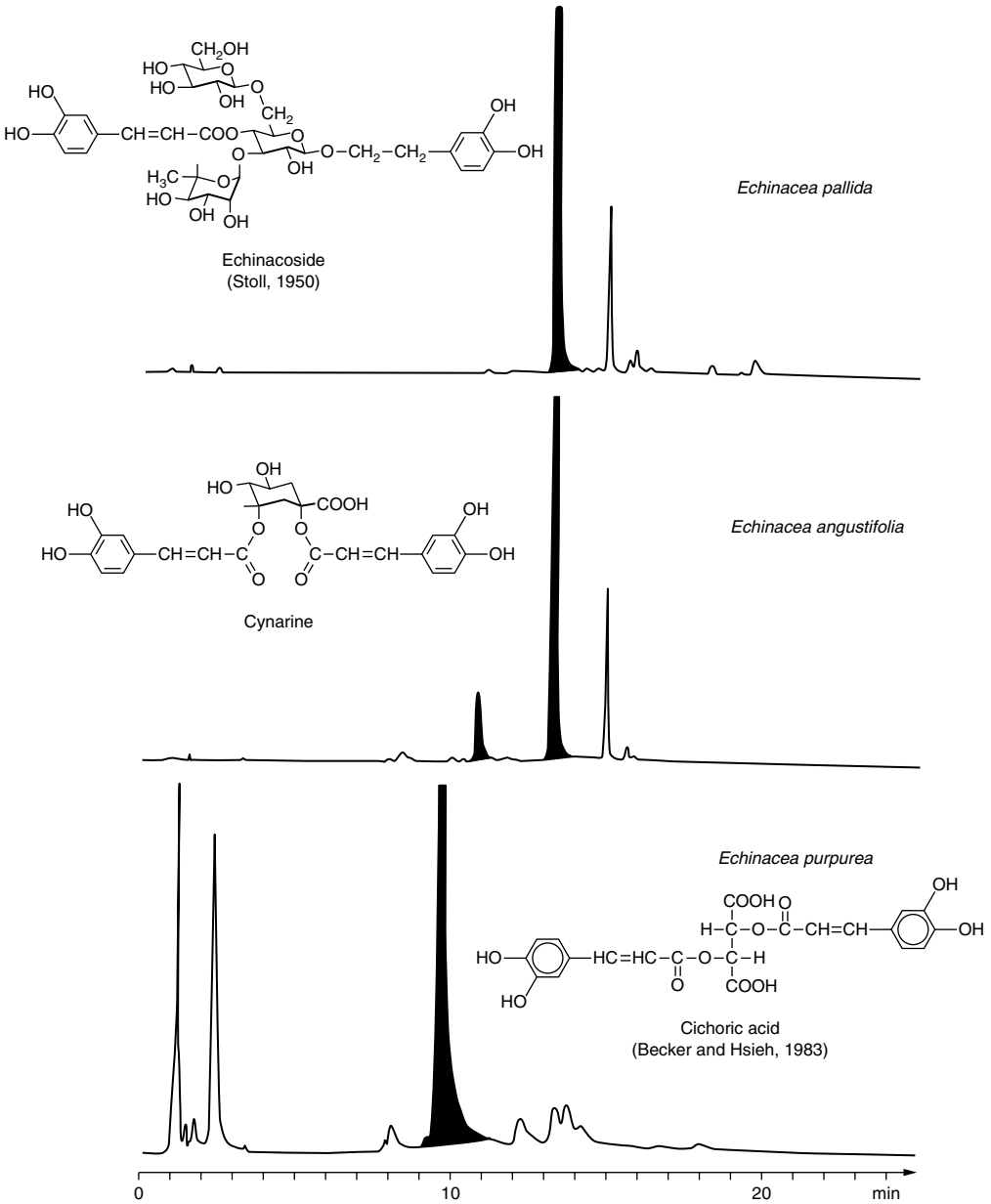

FIGURE 7.3 HPLC separation of caffeic acid derivatives from *Echinacea* roots. (From Bauer, R., 1999, Chemistry, analysis and immunological investigations of *Echinacea* phytopharmaceuticals, in H. Wagner, Ed., *Immunomodulatory Agents from Plants*, Birkhauser Verlag, Basel, pp. 41–48. With permission.)

procedures but similar chromatographic conditions. Among recent procedures the following are relevant: the method proposed by the Institute for Nutraceutical Advancement (INA) and the method described by Bergeron et al. (2000). According to the latter study, ultrasonic extraction of dried roots and aerial parts of *E. angustifolia* and *E. purpurea* with methanol:water (7:3) or ethanol:water (7:3) gave good yields of echinacoside from *E. angustifolia*, of cichoric acid from *E. purpurea* in combination with alkamides 8/9 from *E. angustifolia* and *E. purpurea,* and of alkamide 1 from *E.*

purpurea (recovery in the range of 80% to 90%). The HPLC separation was performed on a short column, and this allowed reduction of analysis times. The separation of the phenolics echinacoside, cichoric acid, cynarin, and chlorogenic acid was obtained with a mobile phase consisting of 50 m*M* sodium dihydrogen phosphate, pH 2.8 (solvent A) and 1% 0.1 *M* phosphoric acid in acetonitrile (solvent B). The elution profile was a linear gradient: 5% to 25% B in 25 min, maintained at 25% B for 2 min, then reduced to 5% in 1 min, and left to re-equilibrate for 5 min. The flow rate was 1.5 ml/min with detection at 320 nm. A pH of 2.80 was carefully selected to avoid peak tailing and satisfactory resolution of cynarin, cichoric acid, and chlorogenic acid. The separation of these phenolic acids and echinacoside is shown in Figure 7.4. To separate alkamides 8/9 (*E. angustifolia*) and alkamide 1 (*E. purpurea*), a linear gradient of acetonitrile and water rising from 40% to 80% acetonitrile in 15 min, decreasing to the initial 40% in 1 min, followed by 6 min equilibration was used. The flow rate was 1 ml/minute; detection was at 210 and 260 nm, and on-line UV spectra were recorded in the range of 200 to 400 nm. This method allowed discrimination between *E. angustifolia* and *E. purpurea* roots, and permitted evaluation of the content of cynarin, cichoric acid, and chlorogenic acid, as well as alkamides 1, 8/9 in various commercial samples of both species. A very large range of concentration (0 to 28,000 ppm for phenolics and 0 to 10,000 ppm for alkamides) for each of the chosen markers between and within species was evidenced, in agreement with the results previously reported by Bauer (1997).

Perry et al. (2001) adapted the INA method to extract and analyze phenolics in *Echinacea* species. Ground plant material was extracted with ethanol:water (7:3) for 15 minutes on an orbital shaker. The extract was then centrifuged and filtered through a 0.45 µm PTFE filter. HPLC separations were performed on a Phenomenex Prodigy column (ODS, 4.6 × 250 mm, 5 µm, 100 A°) with 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient was linear and the profile was the following: from 10% to 22% B in 13 min, to 40% B in 1 min, holding at 0% for 0.5 min, returning to 10% B in 0.5 min and equilibrating for 5 min. The flow rate was 1.5 ml/min and detection was at 330 nm. Under these chromatographic conditions, the elution order was: 2-caffeoyltartaric acid, 7.5 min; chlorogenic acid, 8.2 min; cynarin, 12.2 min; echinacoside, 12.5 min; and cichoric acid, 18.2 min. Chlorogenic acid was chosen as external standard, and each phenolic was quantified as chlorogenic acid. *E. angustifolia* roots had echinacoside as the major phenolic with levels in the range of 0.3% to 1.3% w/w. A mean of 0.08% w/w cichoric

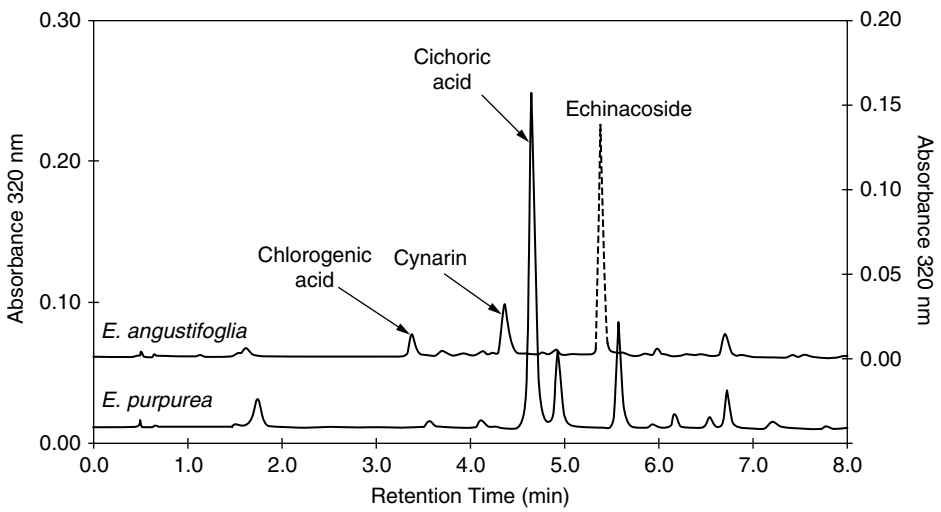

FIGURE 7.4 HPLC separation of caffeic acid derivatives from *E. angustifolia* and *E. purpurea* roots. (From Bergeron, C., et al., 2000, *Phytochem. Anal.*, 11: 207–215. With permission.)

acid was found in the roots, and this result contrasts with the lower levels detected by Bauer et al. (1998) and the higher levels (average of 0.3% w/w) reported by Bergeron et al. (2000). Cynarin was also detected (0.07% to 0.12% w/w), and it was used to distinguish *E. angustifolia* and *E. pallida* roots.

Echinacoside was measured in *E. pallida* roots and the levels averaged 0.34% w/w, which is the low end of the 0.4% to 1.7% w/w range assayed by Bauer et al. (1988). *E. purpurea* had two main phenolics, cichoric and caftaric acids, whose content was in a range similar to that reported by Bauer et al. (1988), at 0.50% to 2.27% w/w and 0.18% to 0.82% w/w, respectively.

Alkamides

As previously mentioned, *E. angustifolia* and *E. purpurea* contain different structural types of alkamides, and published HPLC methods are well suited for the characterization and standardization of the two species (Bauer and Reminger, 1989; Perry et al., 1997; Rogers et al., 1998). The determination of alkamides in various parts of these species was performed on different C_{18} columns by isocratic and gradient elution using aqueous acetonitrile as the mobile phase. Figure 7.5 and Figure 7.6 show typical separation of alkamides from *Echinacea* roots. It is apparent that the patterns of alkamides from *E. angustifolia* and *E. purpurea* are different. By contrast, the HPLC trace of *E. pallida* roots is dominated by the presence of ketoalkenes and ketoalkynes 20 to 25, as shown in Figure 7.7. On the other hand, the content of alkamides 8/9 was in the range of 0.009% to 0.151% and 0.004% to 0.039% in *E. angustifolia* and *E. purpurea* roots, respectively (Bauer and Reminger, 1989).

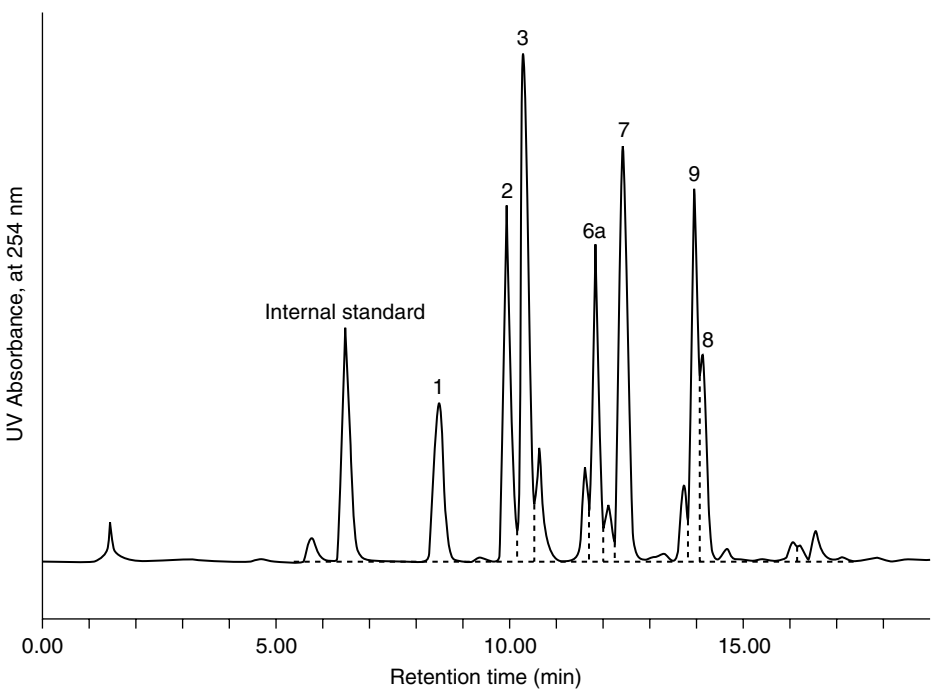

FIGURE 7.5 HPLC trace of alkamides from *E. purpurea* root. Peak 6a, dodeca-2E,4Z-diene -8,10dynoic acid isobutylamide. (From Perry, N.B., 1997, *Planta Med.*, 63: 58–62. With permission.)

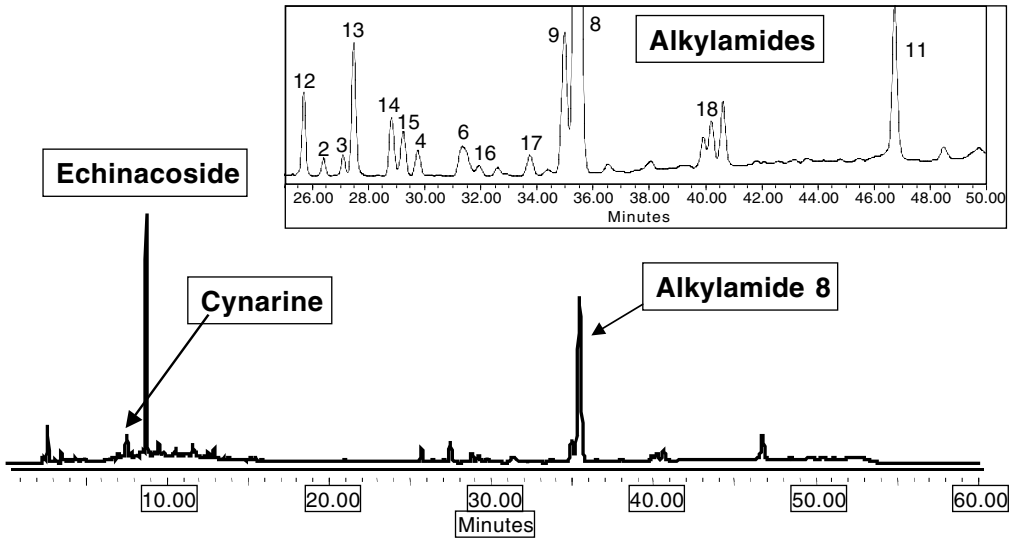

FIGURE 7.6 HPLC trace at 235 nm of an alcoholic extract from *E. angustifolia* root. (From Fuzzati, N., unpublished data.)

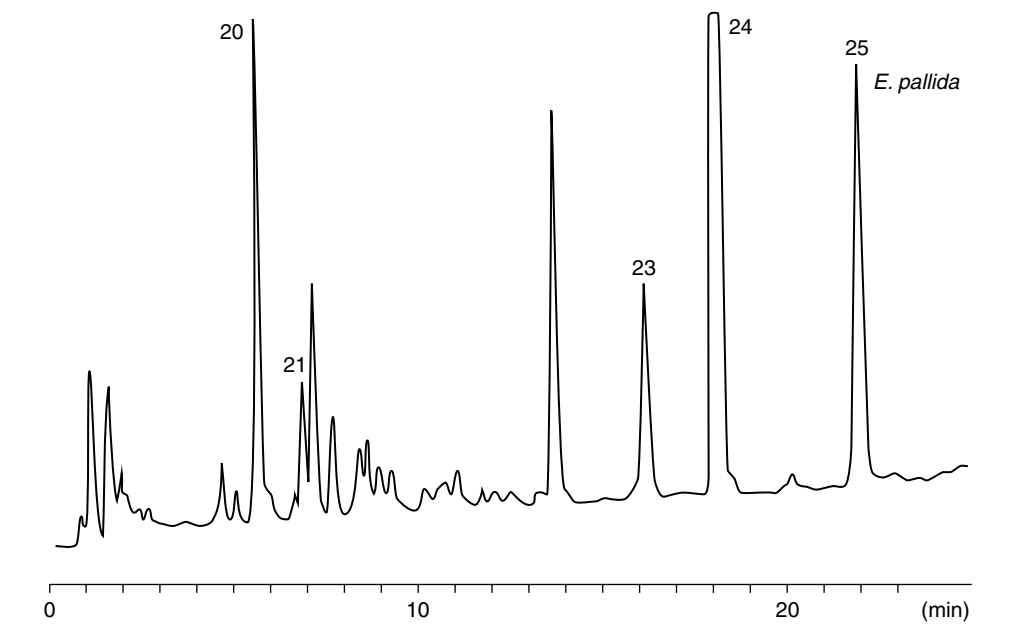

FIGURE 7.7 HPLC trace at 210 nm of ketoalkenes and ketoalkynes from *E. pallida* roots (From Bauer, R. and Reminger, P., 1989, *Planta Med.*, 55: 367–371. With permission.)

European- and U.S.-grown *E. purpurea* roots had 0.004% to 0.039% of the main 8/9 tetraene alkamides, whereas Australian-grown *E. purpurea* roots had 0.013% to 0.102% of 8/9 and 0.024% to 0.394% of total alkamides (calculated as the sum of alkamides 1, 2, 3, 6, 8 and 9). The highest

content of 8/9 was found in New Zealand–grown *E. purpurea* roots (0.17%), rhizomes (0.57%), and vegetative stems (1.41%) (Perry et al., 1997). In leaves and flowers, the content of the same pair was about 0.02% and 0.27%, respectively. The high levels in the perennial (roots and rhizomes) and in the growing parts (vegetative stems) of the plants are justified by the insecticidal activity of alkamides, which protect against herbivorous insect larvae.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY

The different distribution of caffeic acid derivatives among the three species of *Echinacea* was investigated by micellar electrokinetic chromatography (MEKC) (Pietta et al., 1998). This technique (Pietta, 1997) is a mode of capillary electrophoresis (CE), in which surfactants are added to the running buffer. Usually sodium dodecyl sulfate (SDS) is added to the buffer to form negatively charged micelles, which in spite of their charge are forced to migrate toward the cathode by the driving force in CE, that is, the electro-osmotic flow. The analytes move also to the cathode, but at a rate depending on the charge and interaction with the SDS micelles. Thus, MEKC combines both advantages of capillary electrophoresis and reversed-phase chromatography, resulting in higher resolution.

MEKC separation of caffeic acid derivatives were performed by means of a ^{3D}CE system equipped with a diode array detector, using an uncoated fused-silica capillary (58 cm × 50 μm i.d.) with a 3D extended-bubble cell. The running buffer was 25 m*M* tetraborate at pH 8.6, which contained 30 m*M* SDS. The injections were by positive pressure (50 to 100 mbar × seconds, corresponding to about 1 to 4 nl); voltage was 20 kV; the temperature was 30°C and detection was at 320 nm. Under these optimized conditions, characteristic fingerprints of each species were obtained. Typical electropherograms from *E. angustifolia* roots and herb are shown in Figure 7.8. The electrophoretic trace of *E. angustifolia* shows echinacoside as the main phenolic, followed in order by chlorogenic acid, isochlorogenic I acid, and cynarin. The latter two are specific to *E. angustifolia* roots. Echinacoside is also the main compound in *E. pallida*, but the specific constituents are 6-O-caffeoylechinacoside and isochlorogenic acid II. Finally, the electropherogram of *E. purpurea* is dominated by the presence of cichoric acid and caftaric acid, both typical of this species. MEKC was applied also to identify each species even in combination, as exemplified in Figure 7.9.

GAS CHROMATOGRAPHY–MASS SPECTRAL ANALYSIS

Lipophilic constituents, such as terpenes, polyenes, ketoalkenes, ketoalkynes, and alkamides, from the three *Echinacea* species can be analyzed also by gas chromatography coupled to mass spectrometric detection. Lienert et al. (1998) extracted the roots by different methods, namely Soxhlet extraction, maceration, and supercritical fluid extraction, and the resulting extracts were analyzed by means of an HP 5890 gas chromatograph coupled to an HP 5791 MSD mass spectrometer. The capillary column was a CP-wax 56CB; the oven temperature was 55°C for 3 minutes, followed by a temperature ramp at 4.5°C/minute to 230°C, at which the temperature was held for 10 minutes. The mass spectrometer was used in scan mode with an ionization voltage of 1400 eV. Each *Echinacea* species provided a characteristic chromatogram, permitting an easy discrimination between species (Figure 7.10). Interestingly, the extracts obtained by different extraction procedure gave similar GC profiles with a slight difference for extraction yields. Based on these results, Lienert et al. (1998) suggested maceration with a mixture of dichloromethane:pentane (1:1, v/v) for a fast routine analysis of numerous samples.

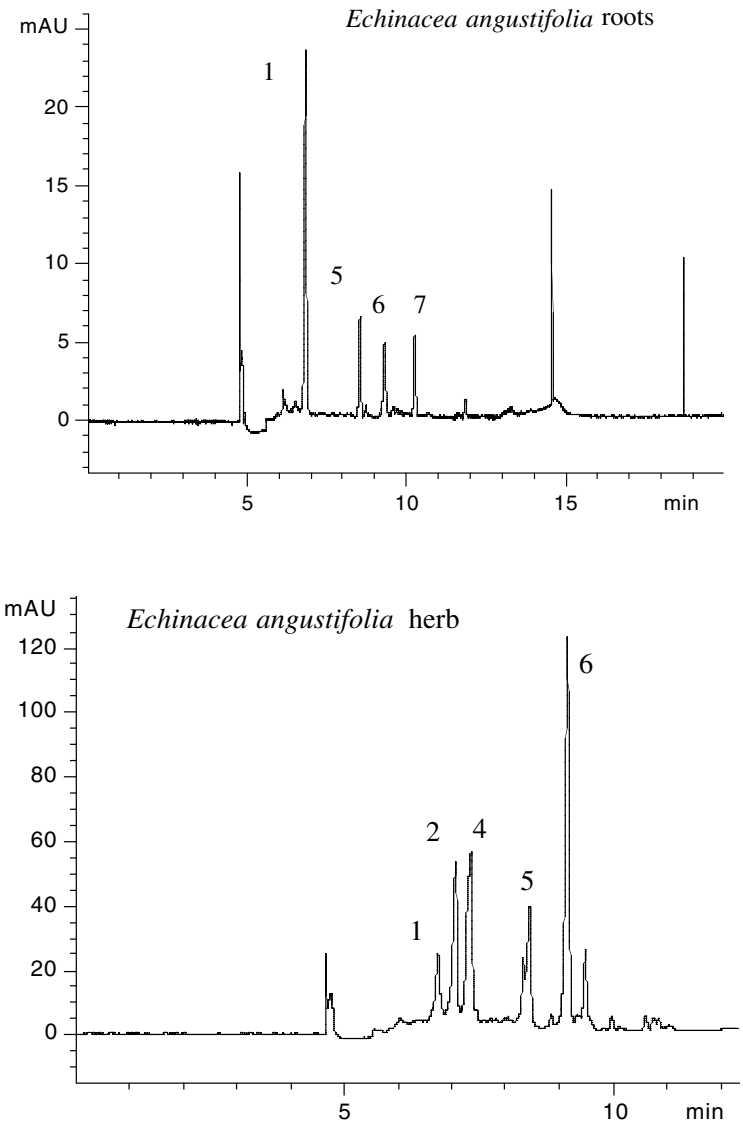

FIGURE 7.8 MEKC electropherogram of methanolic extracts of *E. angustifolia* roots (a) and herb (b). Peaks: echinacoside (1), verbascoside (2), rutin (4), chlorogenic acid (5), isochlorogenic acid I (6), and cynarine (7). (From Pietta, P.G. et al., 1998, *Planta Med*., 64: 649–652. With permission.)

HPLC WITH ULTRAVIOLET AND MASS SPECTROMETRIC DETECTION (HPLC-UV-MS)

High-performance liquid chromatography coupled to UV photodiode array and mass spectrometry has been shown to be a powerful analytical tool for the identification and quantitation of different phytochemicals in complex mixtures. The identity of the analytes is based on the UV and mass spectra obtained by on-line ultraviolet absorbance followed directly by mass spectrometry. However, it must be remembered that mass spectra will not always unequivocally identify

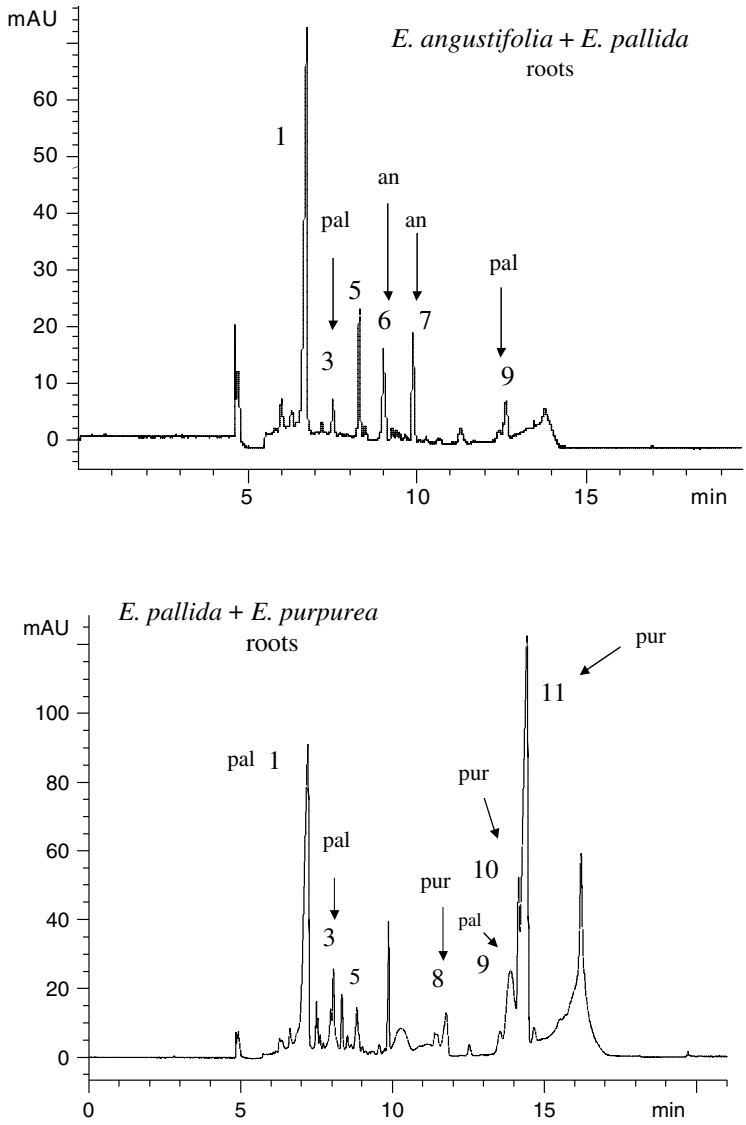

FIGURE 7.9 Electropherogram of *E. angustifolia* and *E. pallida* root (a) and *E. purpurea* and *E. pallida* root (b) mixtures. The arrows evidence peaks specific to *E. angustifolia* (an), *E. pallida* (pal), or *E. purpurea* (pur). Peaks: echinacoside (1), 6-O-caffeoyl-echinacoside (3), chlorogenic acid (5), isochlorogenic acid (6), cynarine (7), caffeic acid (8), isochlorogenic acid II (9), 2-caffeoyl-tartaric acid (10), and cichoric acid (11). (From Pietta, P.G. et al., 1998, *Planta Med.*, 64: 649–652. With permission.)

a particular component, that is, isomers that provide identical spectra. The use of authentic standards for comparing elution times and spectra is required for unequivocal structural identification.

Among the different ionization techniques, the electrospray (ESI) and thermospray ionization (TSP) mass spectrometry appear particularly well suited, since these involve soft ionization that produces in most cases only protonated molecular ions $[M + H]^+$ without fragmentation.

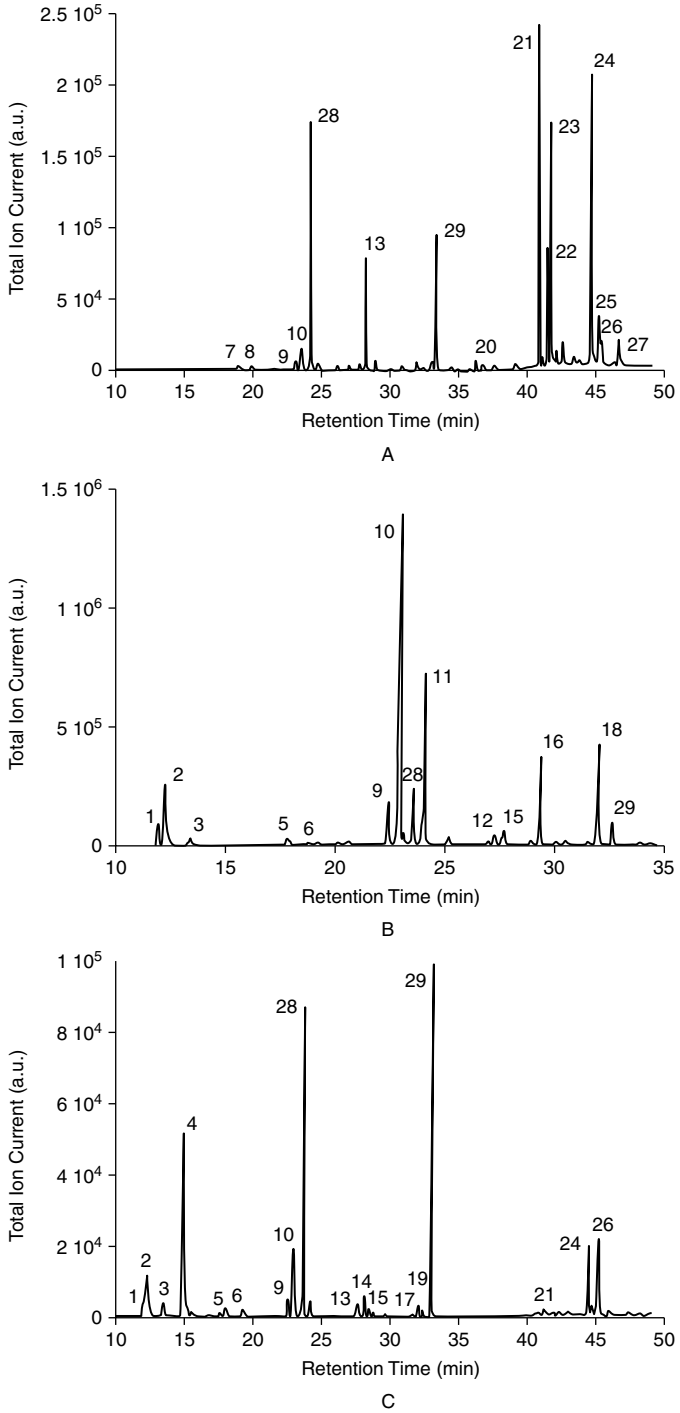

FIGURE 7.10 Typical chromatograms obtained by GC-MS of *E. angustifolia* (top) *E. pallida* (middle), and *E. purpurea* (bottom) roots extracted by maceration with dichloromethane:pentane (1:1, v/v). (From Lienert, D. et al., 1998, *Phytochem. Anal.*, 9: 88–98. With permission.)

HPLC coupled to diode array detection and followed by ESI- or TSP-MS has been successfully applied to discriminate between the three *Echinacea* species. The first paper was published by He et al. (1998), who succeeded in detecting nine alkamides in the roots of *E. purpurea*, and several other alkamides not previously described. The HPLC analysis was performed on a Waters Symmetry C_{18} column (150 × 2.1 mm, 5 µm) using water (eluent A) and acetonitrile (eluent B) as mobile phases by the following elution profile: 0 to 30 minutes, 45% to 80% B; 30 to 32 minutes, 80% to 100% B; 32 to 35 minutes, 100% to 45% B. The flow rate was 0.2 ml/minute and the temperature was set at 45°C. UV spectra were taken in the range 200 to 500 nm; the MS spectra were acquired in the positive ion mode by an electrospray ionization interface, and the mass ranges were 200 to 700 m/z. As shown in Figure 7.11, nine alkamide peaks (Table 7.1) were well separated, while the isomeric pair 8/9 was not resolved. Most peaks yielded protonated molecular ions, sodiated molecular ions, and sodiated molecular dimer ions, exemplified as peaks (Figure 7.12). Purified alkamides 8/9 were employed as external standards to determine the content of these tetraenes in *E. purpurea* root (0.037%) and in *E. pallida, E. purpurea*, and *E. angustifolia* achene samples (0.08%, 0.75%, and 1.06%, respectively).

Sloley et al. (2001) applied the HPLC-UV-MS approach for the rapid characterization of alcoholic extracts from roots and leaves of *E. pallida, E. purpurea*, and *E. angustifolia.* Chromatographic separations were accomplished by gradient elution on a Zorbax 300 SB-C_8 (25 cm × 4.1 mm) column, employing two eluents: A, 0.1% trifluoroacetic acid in 5% acetonitrile; and B, 0.1% trifluoroacetic acid in 70% acetonitrile. The gradient profile was 0 to 20 minutes, from 100% A to 85% A plus 15% B; 20 to 40 minutes, to 100% B; 40 to 45 minutes, 100% B; and 45 to 50 minutes, from 100% B to 100% A. The flow rate was 1 ml/minute. The peaks eluting from the column after UV detection (254 and/or 205 nm) were monitored by an electrospray mass spectrometer. The peaks devoid of strong mass signals (some alkamides) were individually collected and reevaluated by direct injection into the mass spectrometer. According to this study, the major UV-absorbing compounds (254 nm) in *E. angustifolia* roots were alkamides 6 to 9 followed by echinacoside and cynarin. Conversely, *E. pallida* roots had echinacoside and 6-O-caffeoylechinacoside as major UV-absorbing constituents. The chromatogram of *E. purpurea* roots showed mainly cichoric acid.

Leaf extracts from the three *Echinacea* species also provided distinct HPLC UV and electrospray mass spectra profiles. Thus, *E. purpurea* and *E. angustifolia* were differentiated from *E. pallida* for the presence of alkamides 8/9. Cichoric acid was detected in both *E. pallida* and *E. purpurea* but not in *E. angustifolia*. Echinacoside was found in extracts from leaves of *E. pallida* but not in *E. purpurea* and *E. angustifolia* leaf extracts. In addition, rutin was detected in leaf extracts of all three species.

Fuzzati et al. (2001) recently developed an improved HPLC procedure that allows separation of echinacoside and 14 different alkamides from *E. angustifolia* root alcoholic extracts. Interestingly, this method permits satisfactory resolution of the critical isomeric pair 8/9 (Figure 7.6). The eluents are water (A) and 0.01% trifluoroacetic acid in acetonitrile. The elution is on a Zorbax SB C_{18} (250 × 4.6 mm, 5 µm) by a gradient mode. The peaks after UV detection (200 to 500 nm) are fed directly into a mass spectrometer equipped with a TSP-2 interface, and monitored between 200 to 900 m/z in positive ion mode. Similar to ESI-MS, the TSP mass spectra revealed only the protonated molecules ($[M + H]^+$) without fragmentation. This method is currently applied to detect the presence of *E. pallida* roots as adulterant of *E. angustifolia* roots. Indeed, falsification with *E. pallida* roots is easily recognized from the presence of the ketoalkyne pentadeca-8Z,13Z-dien-11-yn-2-one (24), which is specific to this species (Figure 7.13). Finally, echinacoside and alkamide 8 were used to obtain calibration curves for the quantitation of echinacoside and total alkamides (calculated as alkamide 8) in different batches of *E. angustifolia* roots.

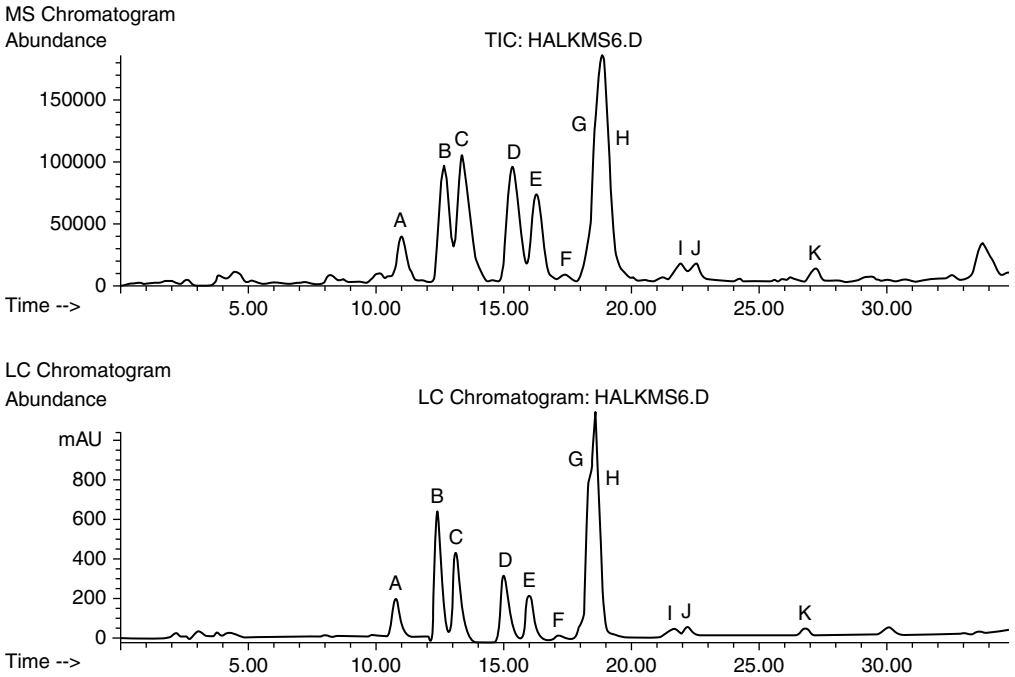

FIGURE 7.11 HPLC and HPLC-ESI-TIC chromatograms of *E. purpurea* roots. (From He et al., 1998, *J. Chromatogr. A*, 815: 205–211. With permission.)

TABLE 7.1
Correlation between Peaks from Figure 7.11 and Alkamides (from Figure 7.1)

Peak	Retention Time (minutes)	Identification
A	10.8	1
B	12.4	2
C	13.1	3
D	15.0	4
E	16.0	6 or 7
F	17.2	15 or 16
G	18.1	8
H	18.5	9
I	21.8	Not identified
J	22.2	10
K	26.9	11

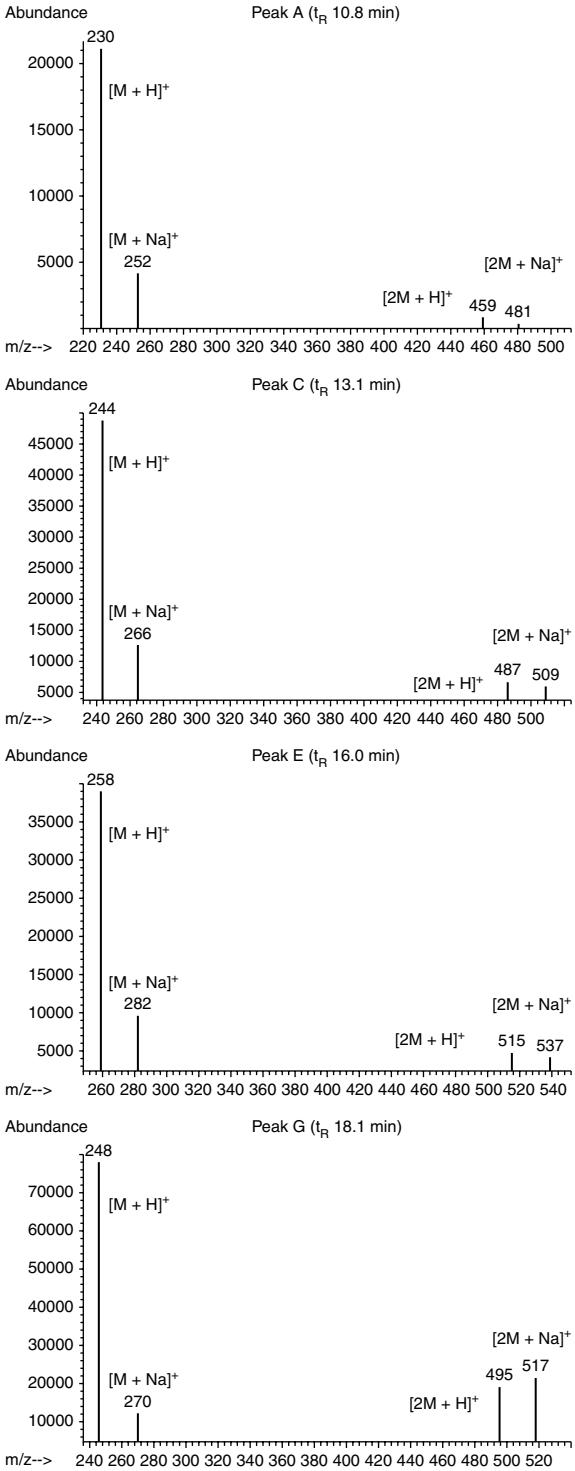

FIGURE 7.12 Mass spectra of some alkamide peaks from Figure 7.11. (From He et al., 1998, *J. Chromatogr. A*, 815: 205–211. With permission.)

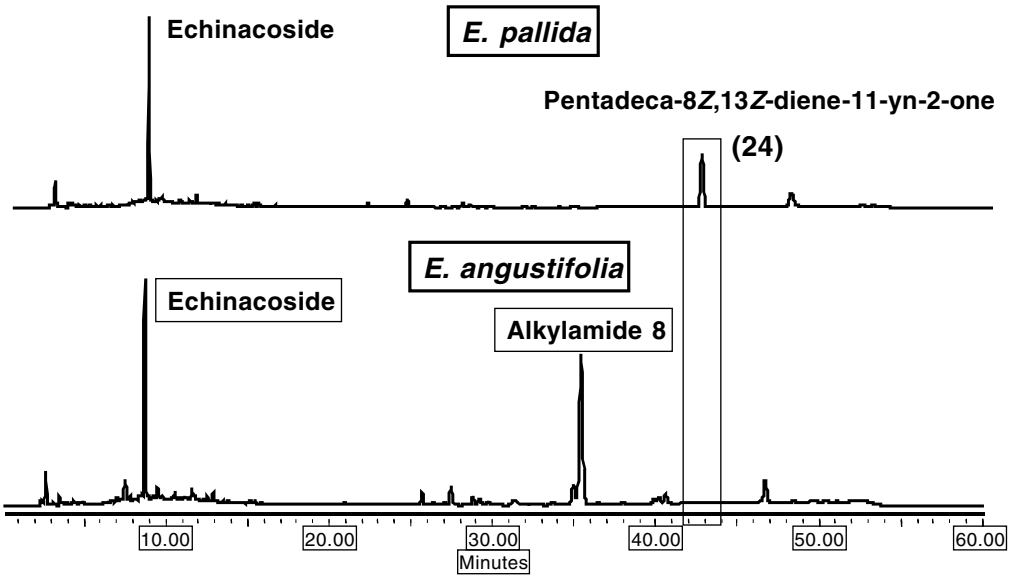

FIGURE 7.13 HPLC traces at 235 nm of alcoholic extracts from *E. pallida* and *E. angustifolia* roots. (From Fuzzati, N., unpublished data.)

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8 Factors Affecting *Echinacea* Quality: Agronomy and Processing

Nigel B. Perry, Ronald B.H. Wills, and Douglas L. Stuart

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References

INTRODUCTION

Echinacea preparations, taken as immunostimulants, are among the top 10 selling herbal medicines in the U.S. and Europe (Bauer, 1998; Brevoort, 1998) with an estimate by Blumenthal (2001) of US$58 million in retail sales in the U.S. in 2000. The three species used in this trade are *Echinacea purpurea*, *Echinacea angustifolia,* and *Echinacea pallida*, with the most popular being *E. purpurea* followed by *E. angustifolia* (McGuffin, 2001). For example, in Australia in 2000, 80 MT of *E. purpurea* were used compared to 15 to 20 MT of *E. angustifolia* and less than 1 MT of *E. pallida* (Walker, 2000). *E. angustifolia* is the most difficult of these species to cultivate but has the highest market value (Binns et al., 2002a; Berti et al., 2002). The scientific literature on *Echinacea* is extensive including major reviews by Bauer (1999a), Hobbs (1994a, 1994b), Mahady et al. (2001) and Wills et al. (2000). In this chapter, the chemistry of the three medicinal *Echinacea* species is summarized with particular focus on the phenolics and alkamides, the bioactive compounds most widely used as quality indicators in *Echinacea*. Aspects examined are quantitative analytical methods for these compounds and their retention during growing, postharvest handling, and pro-

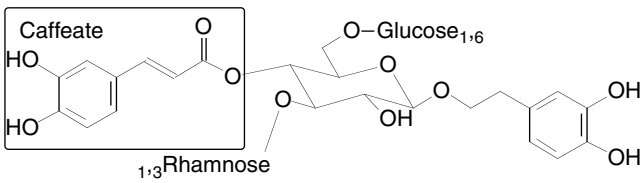
cessing. Literature up to June 2002 is covered, but this review is selective rather than an exhaustive compilation.

BIOACTIVE COMPOUNDS AND ANALYTICAL METHODS

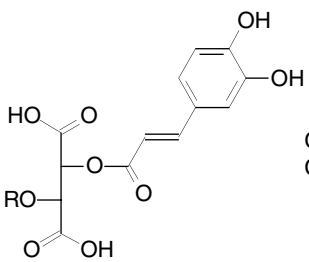
A wide variety of secondary metabolites has been identified in the medicinal *Echinacea* species, including phenolics, alkamides, polyacetylenes, flavonoids, polysaccharides, and various volatile essential oil components (Hobbs, 1994a). Bauer (1999a) concluded that the phenolic cichoric acid, the alkamides, glycoproteins, and polysaccharides contribute to the immunostimulatory activity of *Echinacea* extracts. No routine methods are available for the determination of polysaccharides or glycoproteins (Bauer, 1999a), so we have focused on the phenolics and alkamides as quality indicators.

PHENOLICS

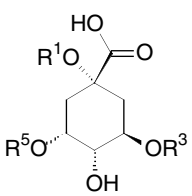
Stoll et al. (1950) reported the first phenolic compound from *Echinacea* as containing catechylethanol and caffeate groups attached to two glucose and one rhamnose sugars. The full structure of echinacoside (Figure 8.1) was determined by Becker et al. (1982). Seven caffeate esters of tartaric acid have been identified from *E. purpurea* (Bauer et al., 1988b; Becker and Hsieh, 1985;



Echinacoside



Cichoric acid, R = Caffeate
Caftaric acid, R = H



Chlorogenic acid, R^1=R^5 = H, R^3 = Caffeate
Cynarin, R^1=R^5 = Caffeate, R^3 = H

FIGURE 8.1 The major phenolic compounds in the medicinal *Echinacea* species.

Soicke et al., 1988), the main ones being cichoric acid (also called chicoric acid) and caftaric acid (Figure 8.1). Apart from its contribution to the immunostimulatory action of *Echinacea* extracts (Bauer, 1999a), cichoric acid has shown activity against HIV (Lin et al., 1999).

The main caffeoyl phenols (Figure 8.1) are useful chemical markers to differentiate between the three medicinal *Echinacea* species (Bauer and Wagner, 1990). Cynarin (1,5-*O*-dicaffeoylquinic acid) is found in *E. angustifolia* roots but not in roots of *E. pallida* or *E. purpurea*, and echinacoside in the roots of *E. pallida* and *E. angustifolia* but not in *E. purpurea* (Bauer, 1999a).

These phenolics all contain several hydroxyl groups, rendering them polar and requiring alcohol:water mixtures to extract them. The caffeate group present in all these compounds provides a characteristic UV absorption, enabling selective detection during HPLC analyses (see below). The caffeate group contains a catechol substructure, which is important for biological activity (Lin et al., 1999), but is also susceptible to polyphenol oxidases, which can lead to instability of these compounds under some conditions (see below).

Alkamides

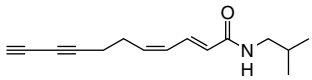
The first report of alkamides (also called alkylamides) of *Echinacea* was by Jacobson (1967) who reported that roots of *E. angustifolia* contained echinacein, assigned the structure dodeca-2E,6Z,8E,10E-tetraenoic acid isobutylamide. A wide variety of alkamides have since been identified from *Echinacea* (Figure 8.2), but none with the structure of echinacein. This structure was probably wrongly assigned by the techniques available at that time. The alkamides are pungent (Jacobson, 1967), being responsible for the tongue tingle caused by some *Echinacea* preparations.

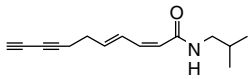
Bauer and Remiger (1989) conducted a comprehensive study of alkamides to determine the difference between *Echinacea* species. They found that *E. purpurea* and *E. angustifolia* have several root alkamides in common, especially the dodeca-2E,4E,8Z,10E and 10Z-tetraenoic acid isobutylamides 8 + 9 (Figure 8.2), which are the most abundant alkamides in both species. Among the other alkamides, the 2,4-dienoic acid unit is present in *E. purpurea* (e.g., 1 to 5) (Figure 8.2), whereas *E. angustifolia* is characterized by the 2-monoenoic acid unit (e.g., 12 to 14) (Figure 8.2). The chromophore of the dienoic acid exhibits a UV absorption maximum at 259 nm, while the monoene maximum is at 210 nm, which is important for HPLC detection by UV (see below). *E. pallida* roots are differentiated from *E. purpurea* and *E. angustifolia* by the presence of 2-ketoalkenes and 2-ketoalkynes (e.g., 20) (Figure 8.2). The aerial parts of the three species show considerable similarity as all contain undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide 1, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides 8 + 9, and dodeca-2E,4E-dienoic acid isobutylamide 11. *E. pallida* has been shown to also contain hexadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide 19 (Bauer and Remiger, 1989). It is important to note that some authors give levels of tetraene alkamides 8 + 9, whereas others report total alkamides. This shows the most difference for *E. purpurea* roots, which contain high proportions of other alkamides (Perry et al., 1997).

The long hydrocarbon chain in all these compounds renders them lipophilic (low polarity), so they do not dissolve in solvent mixtures containing high concentrations of water. The double bonds are susceptible to the same autoxidation processes that lead to rancidity of fatty acids, which may cause instability of the alkamides (see below).

Quantitative Analytical Methods

High-performance liquid chromatography (HPLC) is now the analytical method of choice for the quantitative and qualitative determination of both alkamides and phenolics in the medicinal *Echinacea* species (some recent methods are summarized in Table 8.1). The first step is extraction, and the more polar phenolics are generally extracted with some water present, which is not necessary for the lipophilic alkamides.

1 
undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide

2 
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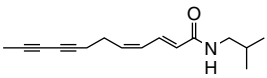
3 
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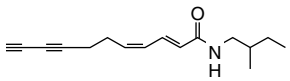
4 
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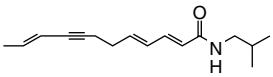
5 
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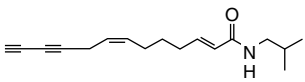
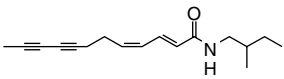
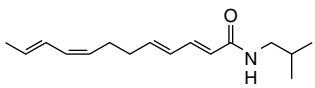
6 
trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide

7 
dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide

8 
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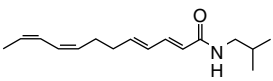
9 
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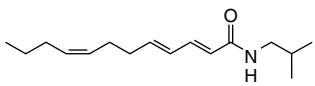
10 
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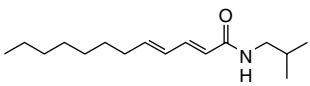
11 
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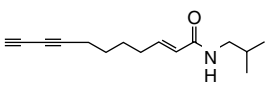
12 
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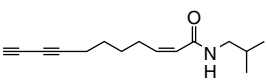
13 
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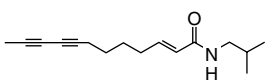
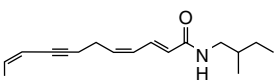
14 
dodeca-2E-ene-8,10-diynoic acid isobutylamide

15 
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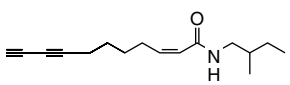
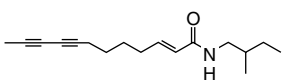
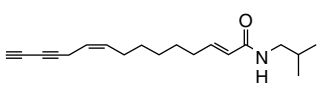
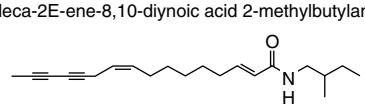
16 
undeca-2Z-ene-8,10-diynoic acid 2-methylbutylamide

17 
dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide

18 
pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide

19 
hexadeca-2E,9Z-diene-12,14-diynoicacid isobutylamide

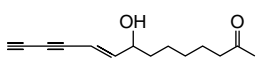
20 
8-hydroxytetradeca-9E-ene-11,13-diyn-2-one

FIGURE 8.2 Alkamides in the medicinal *Echinacea* species.

All HPLC methods used the common reversed phase (RP) C_{18} stationary phase and UV detection. The alkamides generally elute in the same order despite some differences in mobile phase gradients in the different methods (Table 8.1). The phenolics require acidified mobile phase and can vary markedly in retention order (Bergeron et al., 2000). Reference standards of the phenolics are available, but storage and supply of reference samples of the alkamides present problems because of their instability.

TABLE 8.1
HPLC Methods for Analyses of Alkamides and Phenolics in Medicinal *Echinacea* Species

Alkamides

Reference	Extraction	HPLC System[a]	Mobile Phase	Standard	Working Standard	Factor
Stuart and Wills (2000a)	Sonicate; 10 min; MeOH	254 nm; 40°C; 150 × 4.6 mm 5µm	MeCN/H_2O; 1 mL/min; 40% MeCN for 10 min then linear gradient to 53% MeCN at 35 min	Alkamides 8 + 9	Dodeca-2E,4E-dienal	0.978
U.S. Pharmacopeia (2000)	Reflux; 30 min; MeOH	254 nm; 30°C; 250 × 4.6 mm 5µm	MeCN/H_2O (55:45); 1.5 mL/min	Alkamides 8 + 9	Hexa-2E,4E-dienoic acid isobutylamide	1.353
Schieffer (2000)	Shake at 50°C; 60 min; MeOH/H_2O (70:30)	254 nm; ambient; 250 × 4.6 mm 5µm	MeCN/H_2O; 1 mL/min; 50% MeCN to 100% MeCN in 20 min	Alkamides 8 + 9	Alkamides 8 + 9	NA[b]
Livesey et al. (1999)	Reflux; 120 min; MeOH	210 nm; NA; 250 × 4.6 mm 5µm	MeCN/H_2O; 1 mL/min; 40% MeCN to 80% MeCN at 35 min.	Alkamides 8 + 9	Alkamides 8 + 9	NA

Phenolics

Reference	Extraction	HPLC System[a]	Mobile Phase	Standard	Working Standard	Factor
Stuart and Wills (2000a)	Sonicate; 10 min; MeOH/H_2O (80:20)	330 nm; 40°C; 150 × 4.6 mm5µm	Acidified (1% 0.1 M H_3PO_4) MeOH/H_2O; 1 mL/min; 10% MeOH to 50% MeOH at 20 min	Cichoric acid	Chlorogenic acid	0.784
U.S. Pharmacopeia (2000)	Reflux; 15 min; EtOH/H_2O (70:30)	330 nm; 35°C; 250 × 4.6 mm 5µm	Acidified (1% 0.1 M H_3PO_4) MeCN/H_2O; 1 mL/min; 10% MeCN to 22% MeCN at 13 min, to 40% MeCN at 14 min	Chlorogenic acid	Cichoric acid Caftaric acid Cynarin Echinacoside	0.695 0.881 0.729 2.22
Nutraceutical Institute (1999)	Shake; 15 min; EtOH/H_2O (70:30)	330 nm; 35°C; 250 × 4.6 mm 5µm	Acidified (0.1% H_3PO_4) MeCN/H_2O; 1.5 mL/min; 10% MeCN to 22% MeCN at 13 min, to 40% MeCN at 14 min	Chlorogenic acid	Cichoric acid Caftaric acid Cynarin Echinacoside	0.695 0.881 0.729 2.22
Schieffer (2000)	Shake at 50°C; 60 min; MeOH/H_2O (70:30)	330 nm; 35°C; 250 × 4.6 mm 5µm	Acidified (0.3% H_3PO_4) MeCN/H_2O; 1.5 mL/min; 10% MeCN to 22% MeCN at 13 min, to 40% MeCN at 14 min	Chlorogenic acid	Cichoric acid	0.703
Livesey et al. (1999)	Reflux; 120 min; MeOH	320 nm; NA; 150 × 4.6 mm 5µm	0.1N H_3PO_4 in MeCN and 1% 50 µmol/l NaH_2PO_4 (pH 4.5); 1 mL/min; 0-25% MeCN in 20 min	Cichoric acid	Cichoric acid	1

[a] Detection wavelength; column temperature; and column size RP C_{18}.

[b] NA = not available.

BOTANICAL AND CULTIVATION EFFECTS ON BIOACTIVE COMPOUNDS

Differences between Species and Plant Parts

The generally accepted names and taxonomic authorities for the three medicinal species are *E. angustifolia* DC., *E. pallida* (Nutt.) Nutt. and *E. purpurea* (L.) Moench (McGregor, 1968). A taxonomic revision by Binns et al. (2002c) proposes the names *E. pallida* var. *angustifolia* (DC.) Cronq. and *E. pallida* var. *pallida* (Nutt.) Cronq. for the first two taxa. In this review we follow the earlier nomenclature, but the revision does highlight the difficulty of distinguishing *E. angustifolia* and *E. pallida* by appearance (Hobbs, 1994b). Schulthess et al. (1991) have shown that alkamide composition of achenes (fruits) can be used to distinguish the three medicinal species to avoid mistakes in planting, and both alkamide and phenolic composition of roots are distinctive (see above).

Table 8.2 gives levels of the main phenolics and the tetraene alkamides from papers that include quantitative results for at least two of the different species and/or plant parts. Generally these results confirm the qualitative distinctions between the three species made elsewhere (Bauer, 1999a), but the absolute levels reported vary widely.

One possible cause of variation is difference in genotype. The only reported study of natural genotypic variation in *Echinacea* is by Binns et al. (2002a) who cultivated *E. angustifolia* seed from nine wild populations in a controlled environment and compared levels of phenolics and alkamides. The most distinctive population had morphological and chemical characteristics — for

TABLE 8.2
Phenolic and Alkamide Levels in Medicinal *Echinacea* Plant Material (Mean Values, Percent w/w of Dry Plant Material)

Compound	*E. angustifolia*	*E. pallida*	*E. purpurea*		Reference
	Roots	Roots	Roots	Tops	
Caftaric acid	<0.01	0.04	0.3–0.4[a]	0.2–0.8[a]	Perry et al. (2001)
	0.006–0.03[b]	0.004–0.01[b]	0–0.2[b]	0.2[c]	Binns et al. (2002b)
Chlorogenic acid	0.1	0.03	<0.01	<0.01	Perry et al. (2001)
	0.004–0.02[b]	0.008–0.03[b]	0.009–0.2[b]	0.08[c]	Binns et al. (2002b)
Cynarin	0.1	<0.01	<0.01	<0.01	Perry et al. (2001)
	0.04–0.5[b]	0	0–0.008[b]	0[c]	Binns et al. (2002b)
Echinacoside	0.5–1.0	0.5–1.0	ND[d]	ND	Bauer et al. (1988a)
	1.0	0.3	<0.01	<0.01	Perry et al. (2001)
	0.1–0.7[b]	0.02–0.2[b]	0–0.05[b]	0–0.001[c]	Binns et al. (2002b)
	1.3	ND	0	ND	Bergeron et al. (2000)
Cichoric acid	0.09	ND	1.7–2.3[a]	0.5–2.0[a]	Perry et al. (2001)
	0.008–0.06[b]	0.05–0.1[b]	0.5–0.8[b]	0.4–0.9[b,c]	Binns et al. (2002b)
	0	ND	4.0	2.8	Bergeron et al. (2000)
	ND	ND	2.0	2.6	Wills and Stuart (1999)
Tetraene alkamides 8+9	0.5–1.0[b]	0–0.5[b]	0.3–0.5[b]	0.3[c]	Binns et al. (2002b)
	0.2	ND	0.6	0.4	Bergeron et al. (2000)
	ND	ND	0.2	0.03–0.1[a]	Perry et al. (2002).
	0.04–0.07[b]	0	0.1	0.06–0.1[b]	Rogers et al. (1998)

[a] Seasonal range.
[b] Different ages/populations.
[c] In inflorescences.
[d] Not determined.

example, low cynarin levels — which suggested that it resulted from introgression/hybridization between *E. pallida* and *E. angustifolia.* Even for apparently pure *E. angustifolia* populations, the echinacoside levels varied widely, from 0.3% to 0.9% despite averaging analyses of 8 to 17 plants (Binns et al., 2002a). Therefore, there is considerable natural variation that could be used to breed cultivars with higher levels of quality compounds, as described by Fulceri et al. (2001) for *E. pallida*.

Another cause of the variation in quality indicator compounds within a single species is caused by different plant organs having varying levels of secondary metabolites. One *Echinacea* medicinal product, the flowering tops (or aerial parts) of *E. purpurea*, obviously contains the different organs of stems, leaves, and flowers. Even the underground parts of *E. purpurea*, generally called roots, are a combination of rhizomes (underground stems) and true roots. Perry et al. (1997) showed that alkamide levels differed significantly among all of these different parts (Figure 8.3). Bauer et al. (1988b) have shown that cichoric acid levels also vary among aerial parts: 2.2% w/w in flowers, 1.0% in leaves, and 0.4% in stems. Therefore, any agronomic treatments that affect the proportions of plant parts are likely to lead to corresponding changes in the levels of quality indicator compounds.

Cultivation Effects

There are a few published reports on the effects of environmental (including agronomic) factors on the quality indicator compounds of *Echinacea.* Parmenter and Littlejohn (1997) found that *E. purpurea* planting density affected the root:rhizome ratio, which is likely to affect alkamide concentration. Berti et al. (2002) studied the effect of nitrogen, phosphorus, and potassium on root yield, echinacoside, and alkamides in *E. angustifolia* roots. Adding potassium gave significantly higher echinacoside levels (1.04% vs. 0.88% without added K), possibly due to the role of K in sugar translocation to roots, which could be used for synthesis of echinacoside (Berti et al., 2002). Franke et al. (1999) found that different cultivation methods affected *E. pallida* root yield but not the level of echinacoside.

Three separate studies on the effect of growing site and harvest date on levels of phenolics and alkamides in *Echinacea* have been reported from the Southern Hemisphere. Berti et al. (2002) analyzed echinacoside and alkamide levels in *E. angustifolia* roots grown at four sites and harvested on various dates in Chile. Stuart and Wills (2000a) analyzed *E. purpurea* roots and various aerial parts grown at two sites in Australia and harvested at four growth stages for alkamides and cichoric acid. Perry et al. (2002) analyzed *E. purpurea* roots and tops grown at three sites in New Zealand and harvested at five 1-month intervals for alkamides and chicoric acid. Berti et al. (2002) and Perry et al. (2002) did not find any significant changes in alkamide levels, which were highly variable, in *E. angustifolia* and *E. purpurea* roots from different harvest dates and sites. Stuart and

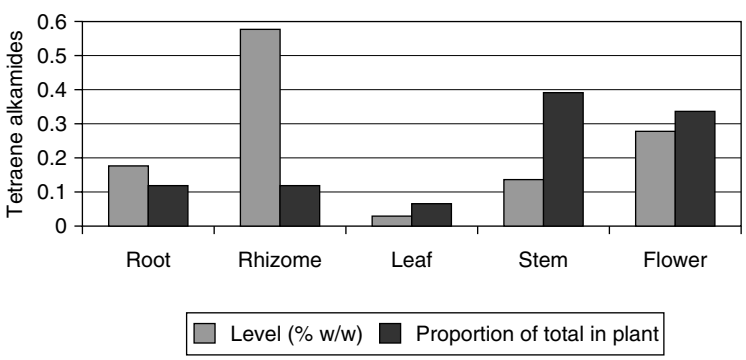


FIGURE 8.3 Tetraene alkamide levels and proportions in different parts of *E. purpurea*. (From Perry, N.B., et al., 1997, *Planta Med.,* 63: 58–62. With permission.)

Wills (2000a) found that alkamide levels in *E. purpurea* roots significantly decreased from the preflowering stage to senescence at both of their sites.

The data from other aspects of these three studies have been combined in Figure 8.4 through Figure 8.6 and show some parallel trends. Both of the studies on cichoric acid in *E. purpurea* roots (Figure 8.4) showed significant differences among sites, and significant decreases at some sites as tops senesced. However, the levels of echinacoside in *E. angustifolia* roots did not change significantly (Berti et al., 2002). The levels of cichoric acid in *E. purpurea* tops (Figure 8.5) showed similar effects with significant differences between sites and significant decreases at some sites as tops senesced. One of the symptoms of senescence is browning of leaves, stems, and flower heads, which could involve enzymatic oxidation of cichoric acid (Kreis et al., 2000). There are significant correlations between cichoric acid levels in tops and roots for both the Australian and New Zealand data, suggesting some sort of translocation among plant parts.

Concentrations of alkamides in *E. purpurea* tops showed significant differences between sites and harvest dates for the New Zealand data (Figure 8.6). This could be ascribed to different flowering times at the various sites, with flowers known to have higher levels of alkamides than leaves or stems (Figure 8.3). However, the late season drop in alkamide levels requires a drop in flower alkamide levels that was not found by Stuart and Wills (2000a) in their senescent flowers. The Australian data therefore showed a different pattern of alkamide variation in *E. purpurea* tops (Figure 8.6)

Clearly, cultivation factors, both growing site and harvest stage, can have major effects on alkamide and phenolic quality indicator levels in *E. purpurea* and *E. angustifolia*, and therefore probably also in *E. pallida*. Medicinal herb growers need to combine these results with data on yields of plant material to enable them to select the optimum harvest time to maximize quality and economic returns.

Proposed Quantitative Standards

Companies in the medicinal herb industry set their own quantitative standards for purchasing *Echinacea* plant material but these vary widely and are considered commercial secrets. The only published quantitative standards are proposed by U.S. Pharmacopeia (2000): for *E. angustifolia* roots, ≥ 0.5% total phenolics and ≥ 0.075% tetraene alkamides. Based on the data presented above, it should be possible to optimize cultivation and harvest to meet the following more challenging standards for high-quality *Echinacea*:

- *E. angustifolia* roots: ≥ 1.0% echinacoside, ≥ 0.5% tetraene alkamides
- *E. pallida* roots: ≥ 0.2% echinacoside
- *E. purpurea* roots: ≥ 1.5% cichoric acid, ≥ 0.2% tetraene alkamides
- *E. purpurea* tops: ≥ 1.5% cichoric acid, ≥ 0.1% tetraene alkamides

However, the levels of these quality indicator compounds in the products that reach consumers, be they encapsulated plant material or extracts, will depend on postharvest handling and processing operations.

POSTHARVEST HANDLING AND PROCESSING EFFECTS ON BIOACTIVE CONSTITUENTS

Despite the well-known lability of many of the active constituents in medicinal herbs, relatively few studies have been conducted to document where losses occur and to optimize handling and processing operations.

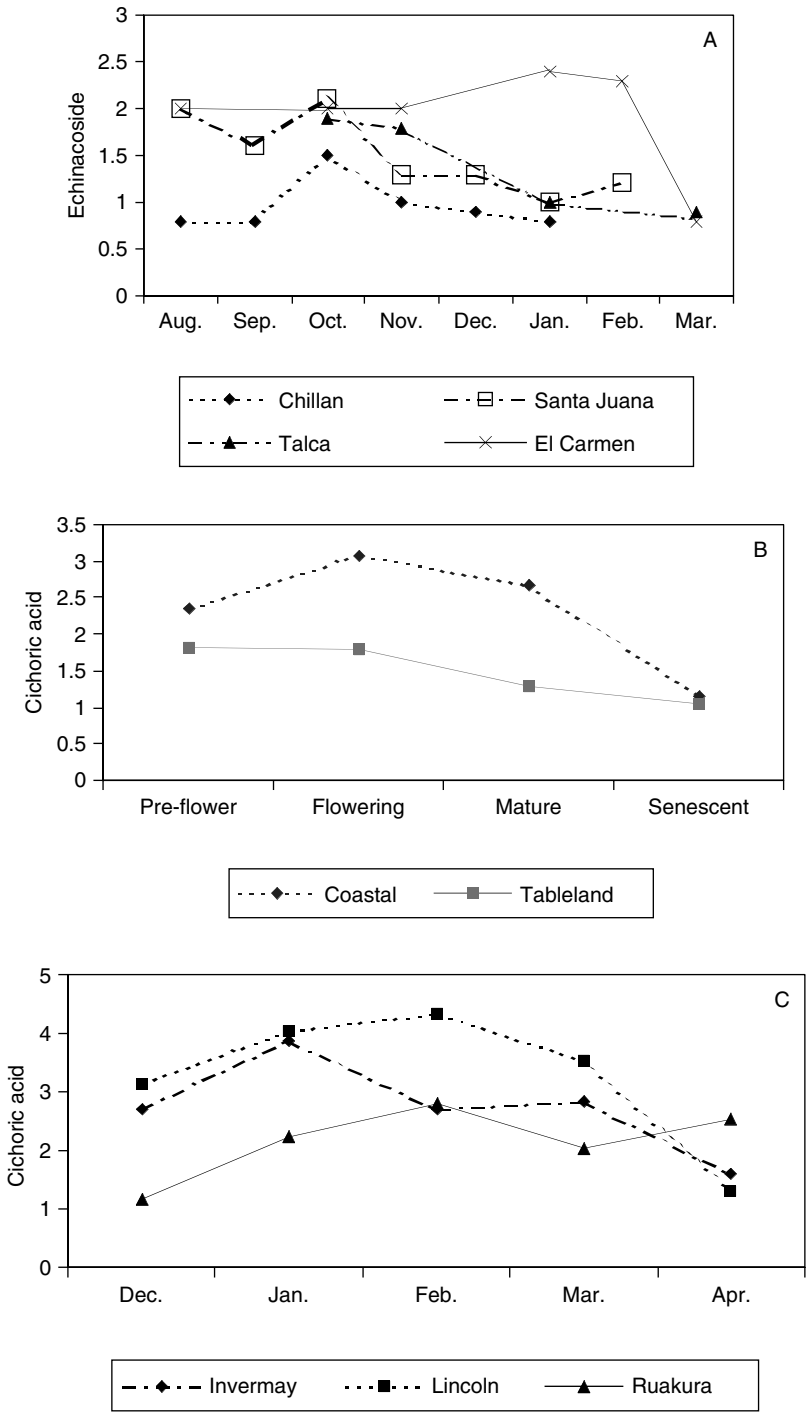


FIGURE 8.4 Phenolics (% w/w) in *Echinacea* roots at different harvest dates and sites. (A) *E. angustifolia* in Chile. (Adapted from Berti, M., et al., 2002, *Acta Hort.,* 576: 303–310. With permission.) (B) *E. purpurea* in Australia. (Adapted from Stuart, D.L. and Wills, R.B.H., 2000, *J. Herbs Spices Med. Plants,* 7: 91–101. With permission.) (C) *E. purpurea* in New Zealand. (From Perry, N.B., et al., 2002, *Echinacea purpurea*: Variation in Yield and Quality between Plant Parts, Harvest Dates and Sites, Crop & Food Research, New Zealand. With permission.)

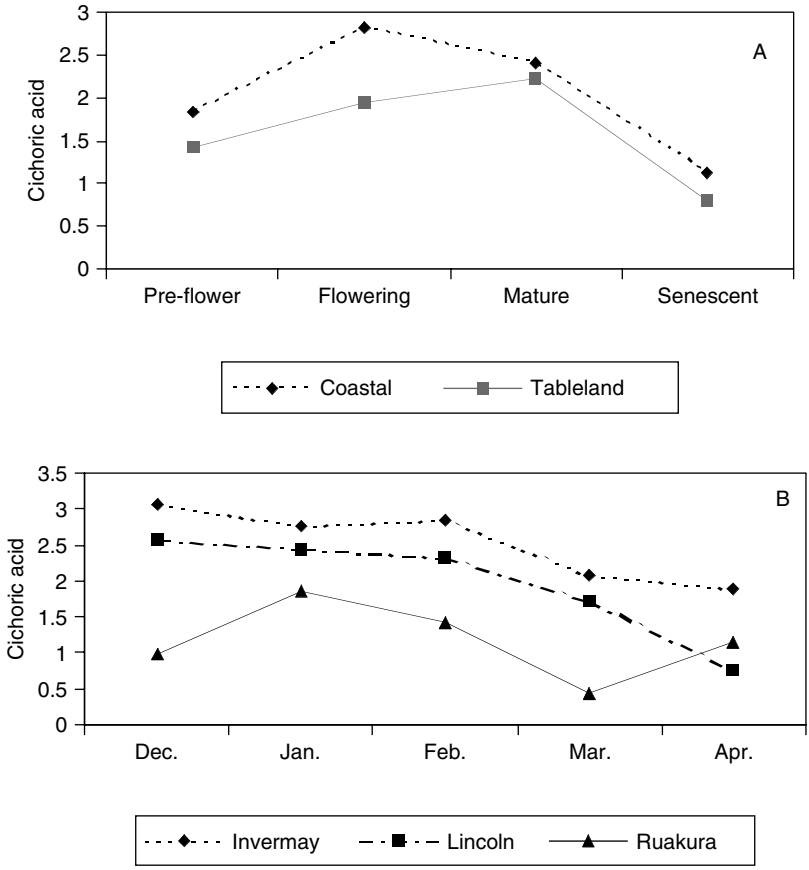


FIGURE 8.5 Cichoric acid levels (% w/w) in *E. purpurea* tops at different harvest dates and sites. (A) In Australia. (Adapted from Stuart, D.L. and Wills, R.B.H., 2000, *J. Herbs Spices Med. Plants,* 7: 91–101. With permission.) (B) In New Zealand. (From Perry, N.B., et al., 2002, *Echinacea purpurea*: Variation in Yield and Quality between Plant Parts, Harvest Dates and Sites, Crop & Food Research, New Zealand. With permission.)

Handling and Drying

The transformation of harvested *Echinacea* plants to a dried product involves a range of handling operations. Wills and Stuart (1999) analyzed 31 root and 31 aerial samples of dried *E. purpurea* offered for sale by Australian growers and found a wide range in the concentration of total alkamides of 0.12% to 1.21% w/w dry weight (1.2 to 12.1 mg/g) in the root samples and 0.02% to 0.39% in aerial samples. Cichoric acid also showed a similar wide range of concentrations at 0.14% to 2.80% and 0.49% to 2.14% in root and aerial samples, respectively. They concluded that most of the variation was due to loss of active constituents arising from suboptimal handling and drying practices. The only other similar published study was by Rogers et al. (1998), also on Australian *Echinacea*, who found a range of 0.02% to 0.11% for total alkamides in nine commercial aerial samples.

Wills and Stuart (2000) found that harvested *Echinacea* plants exposed to varying degrees of physical compression or cutting, and then placed in a drier after 2 and 24 hours showed no significant decrease in the level of cichoric acid compared to undamaged material. It would seem that the level of compression and the cutting resulted in damage to only a small proportion of cells and hence

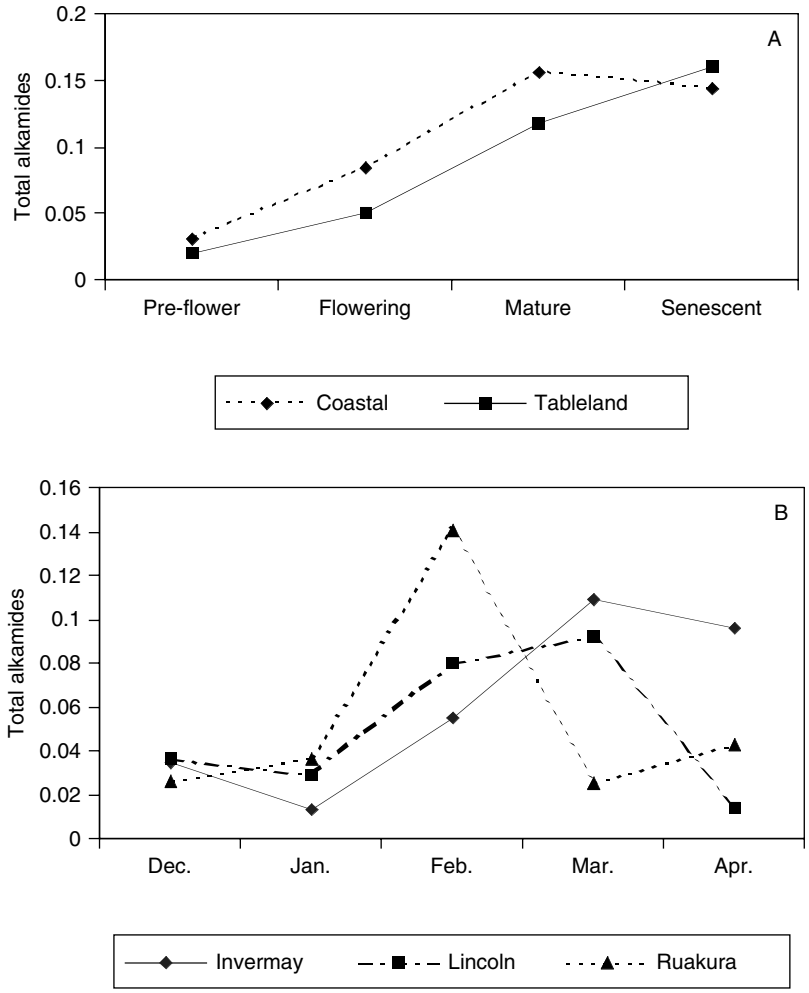


FIGURE 8.6 Total alkamide levels (% w/w) in *E. purpurea* tops at different harvest dates and sites. (A) In Australia. (Adapted from Stuart, D.L. and Wills, R.B.H., 2000, *J. Herbs Spices Med. Plants,* 7: 91–101. With permission.) (B) In New Zealand. (From Perry, N.B., et al., 2002, *Echinacea purpurea*: Variation in Yield and Quality between Plant Parts, Harvest Dates and Sites, Crop & Food Research, New Zealand. With permission.)

there was limited intermixing of substrate with the endogenous cichoric acid oxidative enzymes that were identified by Nusslein et al. (2000). The concentration of alkamides was, however, increased in root material that had been cut into small sections. The increase was attributed to a 50% decrease in drying time compared to that of undamaged plant material and hence a lower total exposure to heat. By contrast, Perry et al. (2000) found that the concentration of total alkamides in chopped and unchopped *E. purpurea* roots was not significantly different after drying, but they exposed all root samples to the same heat loading of about 32°C for 48 hours.

Wills and Stuart (2000) also examined the effect of a substantial time delay between harvest and drying on the retention of alkamides and cichoric acid in undamaged freshly harvested root and flowers stored at 20°C and 60% relative humidity. They found a rapid evaporation of water from both roots and flowers, but no significant decrease in the concentration of alkamides or cichoric acid 10 days after harvest, at which time the plants were commercially dry, that is, ≤ 10% moisture. It is therefore possible to dry *Echinacea* under ambient conditions, provided that the humidity is not so high as to allow microbial growth to affect plant quality.

Stuart and Wills (2003) dried *E. purpurea* root and aerial sections with hot air at 40°C to 70°C and determined the concentrations of alkamides and cichoric acid in the dried products. Increasing drying temperature resulted in a decreased concentration of cichoric acid in all plant sections with a greater loss from aerial plant parts than from the roots. There was, however, no significant difference in the concentration of the alkamides at any drying temperature. The time to reduce the moisture content to 10% decreased from 48 hours at 40°C to 9 hours at 70°C. Establishment of operational parameters for the drying of *Echinacea* must therefore be structured around the more labile cichoric acid.

Kim et al. (2000) found that the level of cichoric and caftaric acids in *E. purpurea* flowers was higher when air dried at 40°C compared to drying at 25°C and 70°C. The highest retention was achieved by freeze drying and vacuum microwave drying, with the latter technique resulting in a very short drying time. Li and Wardle (2001) examined hot-air drying at 35°, 40°, and 45°C of *E. purpurea*, *E. angustifolia*, and *E. pallida* roots. They found that cichoric acid was better retained as the drying temperature increased in *E. purpurea* and *E. pallida*, but no consistent trend was found for echinacoside levels in *E. angustifolia* and *E. pallida*. Of the three species, only *E. purpurea* showed a significant change in water-soluble polysaccharides, with an increase at higher drying temperatures.

Storage

Wills and Stuart (2000) found that the rate of loss of total alkamides from dried *E. purpurea* root and aerial powder stored in the dark increased with increasing storage temperature, with the total loss after 30 days being > 20% at 30°C, 8% at 20°C and 2% at 5°C. Storage at 20°C in the light resulted in a fourfold increase in the rate of loss compared with storage in the dark with > 30% loss after 30 days. The rate of loss of cichoric acid was only substantial at 5°C where > 40% loss occurred after 30 days compared to about 5% at 20°C and < 1% at 30°C, whether stored in the dark or the light. This unexpected decrease in cichoric acid was attributed to substantial absorption of water by the *Echinacea* powder at 5°C, which enhanced enzymatic degradation. This was confirmed with fresh root material that was steam blanched to degrade endogenous enzymes. These samples did not show reduced cichoric acid content when moisture was absorbed.

Perry et al. (2000) examined the effect of extended storage with dried *E. purpurea* root pieces held at −18°C, +3°C, and 24°C, and analyzed for alkamides after 16, 32, and 64 weeks. Loss of alkamides increased with time and temperature. After 64 weeks, roots held at 24°C contained 10% to 20% of the alkamide levels in roots held at −18°C for 16 weeks.

Extraction

Echinacea is commonly available in a range of solid and liquid manufactured products in which concentration of the active constituents from plant material is commonly achieved by solvent extraction. Lienert et al. (1998) showed that extraction of *E. angustifolia* and *E. pallida* with methanol, hexane, and dichloromethane/pentane extracted varying amounts of different alkamides.

In a more comprehensive study using ethanol and water (the most common solvents), Stuart and Wills (2000b) found considerable variation in the yield of total alkamides and cichoric acid from *E. purpurea* root and aerial material under varying processing parameters. The effects, however, differed greatly between the alkamides and cichoric acid. Optimum extraction of alkamides with ethanol/water solutions occurred with 90% to 100% ethanol with a recovery of 70% of alkamides from the plant material. Maximum extraction of cichoric acid was 35%, attained with 60% ethanol, which also gave the best overall yield of active constituents. Increasing the temperature of the extracting solvent from 20°C to 60°C gave decreased yield of alkamides and increased yield of cichoric acid. Increasing the ratio of solvent to substrate increased the extraction of both alkamides and cichoric acid with a doubling of yield as the ratio increased from 2:1 to 8:1. Particle

size had a strong effect on the rate of extraction, with successive doubling of yield for both alkamides and cichoric acid with each decrease in particle size from 2800 to 4000 µm to 1200 to 2800 µm to 300 to 1200 µm. The proportion of individual alkamides was found to be similar to that in the raw material, except for the particle size study where extraction of the largest size (2800 to 4000 µm) resulted in an extract with the tetraene alkamides comprising 55% of total alkamides compared to 30% in all other studies. The Nutraceutical Institute (1999) method for analyzing phenolics in *Echinacea* notes that for efficient extraction and reproducible results, plant materials must be ground to pass through a 400-µm screen.

Livesey et al. (1999) stored 55% ethanolic (liquid) and dried (powder) extracts of *E. purpurea* at −20°C, 25°C, and 40°C for 7 months in sealed containers. They found that the level of tetraene alkamides in the extract was unchanged, but cichoric acid markedly declined at 25°C and 40°C to about 20% of the concentration present at −20°C. The reverse situation occurred with the powder, that is, alkamide levels declined but cichoric acid was unchanged. Stuart and Wills (2000b) found that extracts in 40% to 100% ethanol held in capped jars showed no change in concentration of active constituents over 4 months at 20°C.

Quality of Commercial Products

Analyses of commercial products in three different countries have all shown considerable variations in levels of active constituents. Wills and Stuart (1998) determined total alkamides and caffeoyl phenols — that is, cichoric acid plus echinacoside — due to the presence in some products of *E. angustifolia* in 32 brands of manufactured *Echinacea* products sold in Australia with a label claim to contain *E. purpurea*. The concentration of alkamides ranged from 0.0% to 0.19% w/w (or w/v), and the caffeoyl phenols from 0.0% to 0.83% w/w (or w/v). About 30% of products contained low levels (< 0.02%) of alkamides and 16% had low levels (< 0.03%) of caffeoyl phenols. Expression on a per gram basis of added *Echinacea* showed a similarly large range in concentration of alkamides of 0.0% to 0.38% w/w and for caffeoyl phenols of 0.0% to 1.47%.

Bauer (1999b) analyzed 15 samples of six commercial preparations of expressed juice from aerial *E. purpurea* parts, obtained from German pharmacies, for the tetraene alkamides and cichoric acid. He found considerable variation both between and within commercial batches with the level of alkamides ranging from 0.01% to 0.18% w/v and cichoric acid from 0.0% to 0.38% w/v. Eight samples contained < 0.02% w/v of alkamides and 10 had < 0.01% w/v cichoric acid.

Schieffer (2000) analyzed 35 commercial *Echinacea* products sold in the U.S. He found that the level of total alkamides ranged from 0.003% to 0.44% w/w, and caffeoyl phenols ranged from 0.02% to 2.84% w/w. For products based on a standardized extract, the analyzed constituents were poorly related to label claims.

CONCLUSIONS

The wide variation found in the concentration of alkamides and cichoric acid in commercially traded dry *Echinacea* and in manufactured retail products is of concern because consumers cannot be confident of getting reliable, repeatable clinical effects.

We suggest that the published information reviewed above could be used in the production of *Echinacea* with maximum quality indicated by high levels of bioactive phenolics and alkamides. This will require optimizing choices of chemotype, growing area, and harvest stage; improved handling of the crop before drying and use of more efficient drying technology; and better control over extraction parameters to minimize losses. There is a need for similar studies on *Echinacea* polysaccharides. Upgrading the quality of traded *Echinacea* products may be assisted by the establishment of grading standards for dried *Echinacea* based on the level of active constituents, and by adequate labeling of manufactured products with quality information similar to that required

for the food industry. Clinical trials, which have shown some therapeutic effects from taking *Echinacea* (Melchart and Linde, 1999), must document the species, parts, extraction methods, and standardization compound(s) used so that meaningful comparisons can be made among results.

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9 Popularity, Diversity, and Quality of *Echinacea*

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INTRODUCTION

During the last decade, along with growing interest in CAM (complementary and alternative medicine) therapy and changes in the regulation of dietary supplements, *Echinacea* has become one of the most popular herbal medicines throughout the Western countries, particularly in Europe and in North America, its original source (Asher et al., 2001; Barrett, 2003; Borchers et al., 2000; Kessler et al., 2001; Kligler, 2003). *Echinacea* is also becoming popular in Australia (Wilkinson and Simpson, 2001). In North Africa, South America, and China, people are also paying increasing attention to this herb (Berti et al., 2002; Dou et al., 2001; El-Gengaihi et al., 1998; Hevia et al., 2002; Li et al., 2002; Luo et al., 2003; Shalaby et al., 1997a, b; Wang et al., 2002; Zhang et al., 2001).

The activities of *Echinacea* in modulating the immune system include (1) stimulating *in vitro* and *in vivo* phagocytosis; (2) cytokine production in macrophages; (3) antiviral activity; (4) enhancing natural killer cell activity; (5) inhibiting hyaluronidase, HIV integrase, prostaglandin, and

leukotriene formation; and (6) antioxidant activity (Barrett, 2003; Bauer, 2000). *Echinacea* has been frequently used in preventing and treating uncomplicated upper respiratory tract infections. Its efficacy has been reported in a number of clinical trials, although some results of its effectiveness are inconclusive and inconsistent (Barrett et al., 2002; Bone, 1997a, b; Brinkeborn et al., 1999; Melchart et al., 1998, 2001; Schulten et al., 2001; Schwarz et al., 2002; Turner et al., 2000; Turner, 2001, 2002).

Like many other herbal medicines, it is still not clear how and which of *Echinacea*'s complex range of components exert direct or synergistic effects (Bauer, 1999). This lack of clarity produces difficulty in standardizing plant components and functional end products of *Echinacea*. The situation is further complicated by the fact that different parts (root or the aerial portions) of different species (mainly *Echinacea purpurea, Echinacea angustifolia*, and *Echinacea pallida*) that are cultivated in different areas and harvested at different times have been used in producing various dosage forms with varying strengths (tinctures, juice, tablets, pills, etc.) by diverse preparation procedures that include various extracting solvents (alcohol or water). All of these factors can affect the constituents contained in the raw materials or their end products, and might be the causes of the inconsistent results observed in clinical trials (Bauer, 1999; Dennehy, 2001; Kim et al., 2000a, b; Nusslein et al., 2000; Osowski et al., 2000; Perry et al., 2001; Sloley et al., 2001).

Increasing interest in *Echinacea* is propelling research that will, on the one hand, identify its active constituents and further elucidate its mechanism of action, as well as clarify issues concerning positive/negative indications, most effective doses, and safety, thus guiding a rational use of this herb. On the other hand, it will reveal the diversity in the plant material and in the entire process from planting to the end products (Baugh and Ignelzi, 2000). Therefore, a standardized quality of plant material and end products is doubtlessly needed for such a popular and diversified herbal medicine.

POPULARITY OF *ECHINACEA*

Publications

So far, over 800 scientific studies on *Echinacea* have been published including botanical, chemical, analytical, pharmacological, clinical aspects, and so on. Results of searches for publications about *Echinacea* in the databases Pub-Med, National Library of Medicine (www.ncbi.nlm.nih.gov/PubMed) and ISI Web of Science (www.isinet.com) are shown in Table 9.1 and Table 9.2, respectively.

Although *Echinacea* has a long tradition as a folk medicine among Native Americans and is now the most popular herb in North America, research on *Echinacea* in these countries was rare until the 1990s. Before the 1980s, most research on *Echinacea* was pioneered and conducted in Germany and published in German (Bauer and Wagner, 1991). More recently, studies on *Echinacea* have been carried out worldwide and are published mostly in English (Table 9.1 and Table 9.2).

Scientific publications about *Echinacea* are increasing rapidly. The annual number of publications on this herb found in Pub-Med in 2002 is 50 to 80 times greater than during the 1970s and 1980s. Searching the database CAplus (stneasy.fiz-karlsruhe.de) also showed that studies on *Echinacea* are increasing rapidly. During the 30 years between 1967 and 1997, 131 publications related to *Echinacea* were found (4.4/year), while in the 4 years between 1997 and 2001, more than 200 publications were found (50/year), even though a number of publications were not included in this database. This increasing frequency is just a beginning, and it can be predicted that more studies will be published as the exciting results of new research work are revealed (Binns et al., 2002; Currier and Miller, 2002; Gan et al., 2003; Goel et al., 2002; Pomponio et al., 2002; Speroni et al., 2002).

While searching the Internet with three of the most popular search engines, we encountered duplicated Web pages and advertising. However, by carefully setting the search terms, it is possible to find a large quantity of useful and scientific information. Among these search engines, Google is a satisfactory one in obtaining valuable information on *Echinacea.* The number of Web pages

TABLE 9.1
Publications about *Echinacea* Found in Pub-Med Database Search, 1970 to 2002

	English	German	Others	Total	German (%)	Publications/Year
1970–1979	2	4	1	7	57.1	0.7
1980–1989	6	6	0	12	50	1.2
1990–1999	54	9	3	66	13.6	6.6
2000	33	1	3	37	2.7	37
2001	41	1	1	43	2.3	43
2002	53	2	0	55	3.6	55

TABLE 9.2
Publications about *Echinacea* Found in ISI Web of Science Search, 1961 to 2002

	German	Total	German (%)	Publications/Year
1961–1970	3	4	75	0.4
1971–1980	0	5	0	0.5
1981–1990	9	35	25.7	3.5
1991–2000	6	160	3.8	16
2001	2	46	4.3	46
2002	0	76	0	76

TABLE 9.3
Web Pages about *Echinacea* Found via Major Search Engines, August 2001 and 2002

Search Engine	08/30/2001	08/30/2002	Annual Increase (%)	Pages/Year
Google: www.google.com	167,000	269,000	61.1	102,000
Alta Vista: www.altavista.com	57,346	126,954	121.4	69,608
Lycos: www.lycos.com	90,805	540,421	495	449,616

related to *Echinacea* found when using some search engines has reached 100,000 to 450,000 per year (Table 9.3). It can be seen that the popularity of *Echinacea* is dramatically increasing along with the rising popularity of CAM worldwide.

Cultivation

The current areas of cultivation of *Echinacea* now extend beyond North America and Europe, into South America (Berti et al., 2002), Australia (Walker, 2000), and other areas of the world. Even in North Africa, *Echinacea purpurea* has been cultivated successfully in Egypt (Shalaby et al., 1997a, 1997b). In China, *E. purpurea* has been introduced in the areas of Beijing, Nangjing, and Shanghai (Xiao, 1996), and high-quality plants have been harvested in the Beijing area (Dou et al., 2001). In 2001, the global cultivation area of *Echinacea* was roughly estimated at several thousand hectares (Commonwealth Secretariat, 2001).

Consumers

In Canada, an investigation into the use of herbal products showed that the most popular herbal product recommended by both medical doctors and naturopaths was *Echinacea* (Einarson et al., 2000). According to a national consumer survey conducted in 1999 by Gallup Canada, 33% of the persons surveyed believed that *Echinacea* was a good way to treat the common cold. The Nonpre-

scription Drug Manufacturers Association of Canada published a survey in 1999 to evaluate the consumption patterns and healthcare behavior of 8,000 consumers. Survey results indicated that consumption of herbal products rose from 15% to 35% (from 1996 through 1998). Garlic and *Echinacea* were the most popular self-care herbs (Saskatchewan Nutraceutical Network, 2001).

An estimated 83 million U.S. consumers use CAM (Gertz and Bauer, 2001). Of all CAM treatments, herbal medicine has grown the fastest and *Echinacea* is one of the six top-selling herbal medicinal products (Ernst, 2002). Surveys in the U.S. have shown that more than 7.3 million Americans are using *Echinacea*, and that herbal medicine usage increased from nearly 3% of the population in 1991 to over 37% in 1998 (Briskin, 2000). A dietary supplement survey of 70 pharmacists in the U.S. showed that a majority (53%) of pharmacists reported taking dietary supplements in which *Echinacea* is the top item for colds and influenza (Howard et al., 2001). In another survey determining the frequency of CAM use in surgical patients, results showed that 1,003 of 2,560 patients used CAM, of which herbal medicine (*Echinacea* among the most frequently used) was the most common, primarily for general health maintenance (Leung et al., 2001).

In Germany, physicians prescribed *Echinacea* over 2.5 million times in 1994 (Foster, 2000) and more than 2 million prescriptions for *Echinacea* were filled each year (Kemp and Franco, 2002). In Australia, it is reported that 50% of the population use CAM, of which *Echinacea*-containing products are increasingly popular (Mullins and Heddle, 2002). Annual Australian consumption of *E. purpurea* is approximately 80 MT; dried *E. angustifolia* root, 15 to 20 MT; and *E. pallida*, 1 MT (Walker, 2000).

Market

In 1998, *Echinacea* was the tenth most important medicinal plant sold in Europe with annual sales of about $120 million (Commonwealth Secretariat, 2001). The largest *Echinacea* market in Europe is in Germany where scientists led research on *Echinacea* research throughout the 20th century (Barrett, 2003).

In North America, *Echinacea* is listed as the first among 11 top-selling herbal extracts and among the 12 top-selling bulk herbs (Manitoba Agriculture and Food, 2001). *Echinacea* ranked as one of the best-selling herbal remedies sold in the United States, accounting for 12% of all herbal sales in 1997 (Bent and Avins, 1999). The annual sales of *Echinacea* in the U.S. are estimated to range from more than $200 million (Blendon et al., 2001) to more than $300 million (American Herbal Products Association, 1999; O'Hara et al., 1998; Weil, 1999).

However, the sales of *Echinacea* in 2000 and 2001 declined about 20% in the U.S. (Blumenthal, 2001, 2002). The recent "Product Profile: Medicinal Plants" (International Trade Centre, 2001) indicated that the current trend is oversupply. International markets are overstocked with raw materials, leading to consistently falling prices over the past 2 years. This is particularly true of the main medicinal herbs such as *Echinacea,* which have been greatly overproduced mainly in the developed countries (International Trade Centre, 2001).

DIVERSITY OF *ECHINACEA*

Echinacea diversity will be discussed in terms of its species, varieties, cultivating stage and regions, plant parts, processing of plant and products, methodology, quality, clinical trials, and legislation. The diversity is shown at the level of the following constituents that are thought to show individual or combined biological and pharmacological activity:

- Lipophilic alkamides (dodecatetraenoic acid isobutylamides and related compounds, also called alkylamides)
- Moderately hydrophilic phenolic caffeoyl derivatives (cichoric acid, cynarin, echinacoside, caftaric acid, chlorogenic acid, etc.)

Lipophilic polyalkynes and polyalkenes
High–molecular weight hydrophilic glycoproteins and polysaccharides including heteroxylans, fructofuranoside, and arabinogalactans.

The lipophilic alkamides and polar phenolic caffeoyl derivatives are considered to be the main pharmacologically active components in *Echinacea* alcohol extracts in which the polar polysaccharides are at very low level. The polysaccharides exist in expressed *Echinacea* juice, aqueous extract, and powdered whole herb. However, their levels in most *Echinacea* preparations and effects on the immune system after oral intake have been disputed (Awang, 1999; Bone, 1997a; Borchers et al., 2000).

Species

Table 9.4 lists the species and varieties of *Echinacea* Moench (Heliantheae: Asteraceae). In the most recent publication, genus *Echinacea* has been reclassified as four species and eight varieties, together with a group of introgressant hybrids. *E. purpurea, E. angustifolia,* and *E. pallida* are revised as *E. purpurea* (L.) Moench; *E. pallida* var. *angustifolia* (DC.) Cronq.; and *E. pallida* var. *pallida* (Nutt.) Cronq. (Binns et al., 2002).

In this chapter, we still use the former general names and discuss *E. purpurea, E. angustifolia,* and *E. pallida,* although certain species or varieties show the highest contents of specific phytochemicals. For example, wild *E. pallida* var. *sanguinea* roots contain the highest level (1.9% dwt) of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides; *E. pallida* var. *tennesseensis* flowers contain the highest level (1.04% dwt) of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides; wild *E. atrorubens* var. *paradoxa* roots contain the highest level (3.3% dwt) of echinacoside; and *E. pallida* var. *sanguinea* flowers contain the highest level (3% dwt) of cichoric acid (Binns et al., 2002).

The phytochemical components of various species have been analyzed and compared in a number of studies (Bauer et al., 1988; Bauer and Wagner, 1991; Baugh and Ignelzi, 2000; Hu and Kitts, 2000; Perry et al., 1997; Pomponio et al., 2002; Sloley et al., 2001). Binns et al. (2002) published the most detailed comparison, and by using reverse-phase high-performance liquid chromatography (HPLC) have shown quantitatively the phytochemical variation in the roots and flower heads of native plant populations in genus *Echinacea.*

The diversity of chemical components and antioxidant capacity in the extracts of Canadian-grown *E. purpurea*, *E. pallida*, and *E. angustifolia* have been reported (Binns et al., 2002; Hu and Kitts, 2000; Sloley et al., 2001). Table 9.5 collectively lists some characteristic chemicals (only

TABLE 9.4
Species and Varieties of Genus *Echinacea*

Binns et al. (2002)	McKeown (1999)	Bauer and Wagner (1991)
E. purpurea	*E. purpurea*	*E. purpurea*
E. pallida var. *angustifolia*	*E. angustifolia* var. *angustifolia*	*E. angustifolia* var. *angustifolia*
E. pallida var. *pallida*	*E. pallida*	*E. pallida*
E. pallida var. *simulata*	*E. simulata*	*E. simulata*
E. pallida var. *sanguinea*	*E. sanguinea*	*E. sanguinea*
E. pallida var. *tennesseensis*	*E. tennesseensis*	*E. angustifoliavar tennesseensis*
E. atrorubens var. *atrorubens*	*E. atrorubens*	*E. atrorubens*
E. atrorubens var. *neglecta*	*E. paradoxa* var. *neglecta*	*E. paradoxa* var. *neglecta*
E. atrorubens var. *paradoxa*	*E. paradoxa* var. *paradoxa*	*E. paradoxa* var. *paradoxa*
E. laevigata	*E. laevigata*	*E. purpurea* var. *laevigata*
	E. angustifolia var. *strigosa*	*E. angustifolia* var. *strigosa*

TABLE 9.5
A Collective View of Some Characteristic Chemicals in Three Medicinal Species of *Echinacea*

Components in Alcoholic Extract	*E. purpurea*		*E. angustifolia*		*E. pallida*	
	Root	Leaf	Root	Leaf	Root	Leaf
Cichoric acid	+++	+	+?	–	+?	+
Echinacoside	–	–	++	+?	++	+
Verbascoside	+?	–	–	+	–	+
6-Caffeoylechinacoside	–	–	+?	–	+	–
Cynarin	–	+	+	–	–	+
Caftaric acid	++	+	–	–	–	+
Alkamides	++	+	+++?	+	+?	–
Polyacetylenes	–?	–	–?	–	++	–

Sources: Data from Bauer, R. and Wagner, H., 1991, *Echinacea* species as potential immunostimulatory drugs, in Wagner H. and Farnsworth, N.R., Eds., *Economic and Medicinal Plant Research*, vol. 5, Academic Press, New York, pp. 253–321; Hu, C. and Kitts, D.D., 2000, *J. Agr. Food Chem.,* 48: 1466–1472; Pietta, P., et al., 1998, *Planta Med.,* 64: 649–652; Sloley, B.D., et al., 2001, *J. Pharm. Pharmacol.,* 53: 849–857.

comparing relative levels) in the three medicinal species of *Echinacea* (Bauer and Wagner, 1991; Hu and Kitts, 2000; Pietta, 1998; Sloley et al., 2001). Although the levels varied according to various factors, in general cichoric acid and caftaric acid are the main caffeoyl derivatives in *E. purpurea* roots. Echinacoside, 6-O-caffeoylechinacoside (Sloley et al., 2001), and some unique polyacetylenes (Bauer and Wagner, 1991) are the components present in extracts of *E. pallida* root. Extracts of *E. angustifolia* roots could be distinguished from those of *E. purpurea* and *E. pallida* by the absence of, or only a trace of, cichoric acid, and by the presence of both cynarin and echinacoside (Sloley et al., 2001). It should be noted that the alkamides differ greatly among species in amounts and in general chemical structure (Bauer and Wagner, 1991, Binns et al., 2002). Alkamides are present in *E. purpurea* and particularly as dodecatetraenoic acid isobutylamides in *E. angustifolia* but not in *E. pallida* (Giancaspro, 2000).

Mazza and Cottrell (1999) have analyzed and identified 70 volatile components in the plant parts of Canadian-grown *E. angustifolia, E. pallida*, and *E. purpurea* (Table 9.6). Some volatile compounds, such as acetaldehyde, camphene, and limonene, are present in all plant tissues irrespective of the species, while some components varied with the species and the plant parts. Table 9.6 shows the relative levels of some volatile compounds in the three species.

Species diversity can be observed also in the alkamide content of the achene. Achenes of the three species were extracted by n-hexane and analyzed by HPLC-UV-ES-MS (He et al., 1998). Their results (Table 9.7) were the same as those reported in an earlier study (Schulthess et al., 1991); the *E. pallida* achene can be easily differentiated from the others by the remarkably low content of isobutylamides in the achene.

Variety and Other Factors

The natural variation of *Echinacea* within a species can have a tremendous effect on final product quality. This diversity might be due to genetic and environmental differences including variety, cultivation regions, harvest time, and cultivation or processing conditions.

In general, the wild *E. angustifolia* has higher echinacoside content than the cultivated one (Berti et al., 2002). Wills and Stuart (1999) analyzed active components in 62 commercial samples of dried root and of aerial parts of *E. purpurea* grown in eastern Australia. Table 9.8 shows the wide range in contents of the two active components in the samples. Results from Table 9.8 showed that *Echinacea purpurea* roots contain more alkamides than the aerial parts.

TABLE 9.6
Several Volatile Components in Three Medicinal Species of *Echinacea*

Components	*E. purpurea*				*E. angustifolia*				*E. pallida*			
	Roots	Stem	Leaf	Flower	Roots	Stem	Leaf	Flower	Roots	Stem	Leaf	Flower
α-Phellandrene	+++	–	–	–	+	–	–	–	–	–	–	–
β-Myrcene	–	+++	+++	++++	–	+++	+++	+++	+	+++	+++	++++
Dimethylsulfide	+++	+	+	+	+	+	+	+	++	+	+	+
α-Pinene	+	++++	+++	++++	+	+	++	+++	–	+	++	++
P-cymene	++	–	+	+	+	–	–	–	–	–	–	–

TABLE 9.7
Achene Content of Alkamides in Three Medicinal Species

Components (mg/g Dry Mass)	*E. purpurea*	*E. angustifolia*	*E. pallida*
Alkamide (dodeca-tetraenoic isobutylamide)	0.75	1.06	0.08

Source: Data from He, X.G., et al., 1998, *J. Chromatogr. A*, 815: 205–211.

TABLE 9.8
Amount Range of Active Components in 62 Samples of *E. purpurea*

Components	Roots (mg/g Dry Weight)		Aerial Parts (mg/g Dry Weight)	
	Mean	Range	Mean	Range
Total alkamides	6.2±2.4	1.2–12.1	1.0±0.7	0.2–3.9
Cichoric acid	13.2±5.0	1.4–18.0	12.9±4.5	4.9–21.4

Source: Data from Wills, R.B.H. and Stuart, D.L., 1999, *Food Chem.,* 67: 385–388.

Methodology and Laboratories

The diversity in results is also due to laboratories using different methodology and conditions. In Table 9.5, the question marks indicate the presence of different results with respect to the component levels from different laboratories using the same species (Pietta et al., 1998; Sloley et al., 2001).

Speroni et al. (2002) and Sloley et al. (2001) reported that echinacoside is present in *E. pallida* and only in traces in *E. angustifolia*, whereas Perry et al. (2001) reported that *E. angustifolia* contained more echinacoside (1.04%) than *E. pallida* (0.34%). As mentioned above, Binns et al. (2002) revealed new levels of some constituents in the root and flower head of wild and cultivated populations of *Echinacea*.

In addition, there are some inconsistencies in the results from studies on immune-regulating activity of *Echinacea* (Borchers et al., 2000). A recent study by South and Exon (2001) concluded that *Echinacea* preparations under some conditions may have immunosuppressive rather than immunostimulating activity. Thus, it is currently argued that characterizing *Echinacea*'s effects as "immunomodulation" may be more appropriate (Barrett, 2003).

Schwarz et al. (2002) reported an unexpected result in a study with a double-blind, placebo-controlled crossover design, in which 40 healthy men (20 to 40 years of age) received 2 weeks of orally administered freshly pressed *E. purpurea* juice or placebo juice. Their study showed that compared with the placebo, *E. purpurea* had no effects in enhancing phagocytic activity of either polymorphonuclear leukocytes or monocytes.

Regions

Biological diversity among *Echinacea* species cultivated in different regions also exists. Studies carried out in China showed that the introduced Canadian *E. purpurea* planted in the Beijing area accumulated more cichoric acid than that planted in Canada (1.108% vs. 0.671%) (Dou et al., 2001). The levels of the major alkamide isomer pair (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide) in *E. purpurea* roots were 0.04 to 0.39 mg/g (Bauer and Remiger, 1989) in Germany and 0.8 to 3.6 mg/g (Perry et al., 1997) in New Zealand. These differences may be caused by the geographical factors, climate, soil, and cultivation conditions, as well as preparation methodology for testing.

Plant Parts

At present, most preparations are derived from the aerial parts of *E. purpurea* and underground parts of *E. purpurea*, *E. angustifolia*, or *E. pallida*. In an individual species or cultivar the different parts of the plant contain different levels of the active compounds (Table 9.5, Table 9.6, and Table 9.8). Several studies have reported that in *E. purpurea*, alkamide levels were much lower in leaves than in roots. Perry et al. (1997) reported that alkamide levels differed significantly among the various parts of *E. purpurea*. Kim et al. (2000a) reported the total alkamide levels in Canadian-grown *Echinacea purpurea*. In roots, the level of total alkamides (2.65 to 3.28 mg/g) is much higher than that in leaves (0.10 to 0.18 mg/g).

Stuart and Wills (2000a) also analyzed the distribution of alkamide and cichoric acid levels in morphological parts of *E. purpurea* grown in Australia and extracted with methanol (Table 9.9). Root is a better source of alkamides, while flower and leaf are better sources of cichoric acid. The major alkamides in *E. purpurea* root are the isomer pair (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide); these two major alkamides account for about 50% of total alkamides (Wills and Stuart, 2000). In Egyptian-grown *E. purpurea*, it was also found that these two alkamides were the major constituents in roots at all stages of development, constituting 46.4% (in fruit stage) to 75.9% (in seedling stage) of total alkamides (El-Gengaihi et al., 1998).

Component Levels at Plant Development Stage

Growth Stage

The accumulation of phytochemicals in *Echinacea* varies with growth stages, species, cultivation conditions, and regions. Binns et al. (2002) have compared and defined the phytochemicals accumulating with age in all Canadian-grown species and varieties of *Echinacea*. Stuart and Wills (2000a) investigated the change in alkamide and cichoric acid levels during the growth stages of Australian-grown *E. purpurea*. During the four growth stages (pre-flower, flowering, mature, senescent), the alkamide level decreased in root, stem, and leaf tissues, but increased in the flower tissue to senescence. At all stages, the alkamide level was higher in the root than in the stem or leaf. The level of cichoric acid showed no significant change during the flowering and mature stage. The cichoric acid level in stems was significantly lower than that in other tissues (Table 9.10). El-Gengaihi et al. (1998) investigated alkamide accumulation in *E. purpurea* cultivated in Egypt; these authors showed that in the roots, but not in the vegetative tissues, alkamides increased and reached a maximum at the plant fruiting stage.

Berti et al. (2002) studied the effects of phonological stages; nitrogen, phosphorous, and potassium fertilization on root yield; and echinacoside and alkamide content in *E. angustifolia* cultivated in Chile. Results showed that echinacoside and alkamides were strongly affected by the phonological stage. Echinacoside and alkamide contents were inversely correlated with root yield. Echinacoside content was proportionally affected by potassium supply.

TABLE 9.9
Active Components Distribution in Parts of *E. purpurea*

Components (%)	Root	Stem	Leaf	Flower
Alkamides (extracted by 100% methanol)	~70	10	1	~20
Cichoric acid (extracted by 80% methanol)	20	10	35	35

Source: Data from Stuart, D.L. and Wills, R.B.H., 2000, *J. Herbs, Spices Med. Plants,* 7: 91–101.

TABLE 9.10
Active Component Levels during Growth Stages

Growth Stage	Components	Tissue (mg/g Dry Weight)			
		Root	Flower	Stem	Leaf
Preflowering	Alkamide	11.7			0.3
	Cichoric acid	23.6			18.4
Flowering	Alkamide	10.2	0.8	1.2	0.3
	Cichoric acid	30.6	32.3	7.6	28.8
Mature	Alkamide	9.5	1.9	0.5	0.1
	Cichoric acid	26.6	30.4	9.0	24.1
Senescent	Alkamide	9.0	1.9	0.5	<0.1
	Cichoric acid	11.5	16.3	8.5	9.8

Source: Data from Stuart, D.L. and Wills, R.B.H., 2000, *J. Herbs, Spices Med. Plants,* 7: 91–101.

Flower Developmental Stages

Letchamo et al. (1999) studied the accumulation of active ingredients during the development of the flower heads of the American-grown *E. purpurea*. The quality of *Echinacea* was strongly influenced by floral development, which was divided into four stages, from early flower buds to the senescent stage. The highest content of cichoric acid was found at Stage 1, and the content of isobutylamide was highest at Stage 3 and 4. The maximum content of chlorogenic acid and echinacoside occurred at Stages 1 and 2, respectively. To obtain optimal yields of both hydrophilic and lipophilic components, *Echinacea* flowers should be harvested at Stage 3.

Processing Conditions

A number of recent studies have showed how varying methods of extraction, drying, and storage affect levels of active components (He et al., 1998; Hevia et al., 2002; Hudaib et al., 2003; Kim et al., 2000a, 2000b; Livesey et al., 1999; Menon et al., 2002; Perry et al., 2000; Stuart and Wills, 2000b, 2003; Sun et al., 2002). All these factors caused diversity in the plant material and final products. Chapter 8 in this book by Perry et al. describes these factors in detail.

As mentioned above, *Echinacea* contains a considerable number of phytochemicals, some of which are water soluble. Therefore, the processing methodology will affect the level of the different components extracted. The level of polysaccharides will be much lower if alcohol extraction is used during preparation.

Brovelli et al. (2001) compared two types of press to make juice from U.S.-grown *E. purpurea*: the hydraulic bag press and a mechanical screw press. The results showed differences not only in physical parameters but also in the chemical nature of the juices. Juice extracted by screw press had twice the concentration of cichoric acid as the juice extracted by the bag press. There was also a qualitative and quantitative difference in the alkamide fraction in favor of the screw press.

Adulteration

Over the last several years, the market for *Echinacea* has grown rapidly. As a result, there has been an increase in species misidentification or adulteration in the *Echinacea* trade. Inadequate quality control means that ineffective or adulterated products can reach the market. The literature and the media have revealed examples of *Echinacea* preparations of poor quality and low amounts of characteristic constituents.

Roots of *Parthenium integrifolium* L., commonly known as American feverfew, have been found to be adulterants/substitutes for *Echinacea* root (Turner, 2001). Its roots are larger and easier to harvest than *Echinacea* roots. This adulterant/substitute can be recognized by the absence of any caffeoyl derivatives (Giancaspro, 2000) or through the presence of the sequiterpene esters cinnamoylechinadiol, cinamoylepoxyechinadiol, cinnamoylechinaxathol, and cinnamoyl dihydroxynardol.

Wolf et al. (1999) described the discrimination of the three main species of *Echinacea* by random amplified polymorphic DNA (RAPD) analysis. Individual *Echinacea* species are easily identified by RAPD analysis. Adulterations due to drug mixtures also can be detected. Laasonen et al. (2002) have developed a near-infrared reflectance spectroscopic method for the fast (analysis within 1 minute) qualitative identification of *E. purpurea* dried milled roots. An adulterated *E. purpurea* sample can be detected at a minimum of 10% adulteration.

Product Quality

The phytochemical studies on *Echinacea* have revealed tremendous diversity in the quality of *Echinacea* products derived from various sources (Bergeron et al., 2000; Gilroy et al., 2003; Letchamo et al., 1999; Weil, 1999). The potency of *Echinacea* products can vary from manufacturer to manufacturer and from lot to lot from a single manufacturer, all of which can be attributed to quality diversity.

Echinacea is available to consumers in many forms, including tinctures, pressed juice, liquid, tablets, pills, powders, capsules, lozenge, beverage, spray, soft gel, ointments, lotions, creams, toothpastes, and teas. In earlier publications, products for the parenteral administration of *Echinacea* existed in Germany (Parnham, 1996). Now, many hundreds of products are available worldwide (Bauer, 1998). Even in Australia, there are hundreds of *Echinacea* products listed in the Australian Register of Therapeutic Goods (ARTG) containing *Echinacea* alone or in combination with other herbs, vitamins, or minerals (Cameron, 1998). Tinctures or extracts of *Echinacea* in alcohol are the form most herbal authorities recommend. In the U.S., the most commonly used preparation is a liquid extract made from the root of *E. purpurea* (Kligler, 2003). In Germany, freshly pressed *E. purpurea* juice is popular (Bauer, 1999).

Different formulations of *Echinacea* preparations may have different contents of active ingredients and exert diverse pharmacological effects in the human body. Products derived from an extract containing more than 50% ethanol are not considered capable of the effects of water-soluble polysaccharides since in this concentration polysaccharides are insoluble (Stuart and Wills, 2000a). Freshly pressed *Echinacea purpurea* juice may contain certain levels of polysaccharides (Bauer, 1999).

Like the other herbal medicines, one problem of quality control and standardization in *Echinacea* products is that many countries have their own regulatory criteria and are not prepared to accept

products from other countries that have been assessed by different criteria. The quality control of *Echinacea* thus varies by country and manufacturer. In the U.S., neither the Food and Drug Administration (FDA) nor any other federal or state agency routinely tests herbal medicines or other dietary supplements for quality prior to sale (Goldman, 2001). It was not until March 1999 that the FDA required that the labeling of herbal products provide information identifying the species of the herb, the part of the plant used, and the concentration of the herb.

Gilroy et al. (2003) investigated 59 single-herb preparations of *Echinacea* purchased from 11 stores in the Denver area over a 2-day period in August 2000. The samples included tablets, capsules, soft gels, and liquid. The results of thin layer chromatography (TLC) analysis showed that six samples (10%) contained no measurable *Echinacea* at all. The concentration of cichoric acid in the samples of the *E. purpurea* species ranged from 0% to 1.46%. In addition, the recommended daily dose of these samples ranged from 45 to 5,380 mg while German Commission E recommends a daily dose of 900 mg. The price per recommended dose ranged from $0.02 to 2.99.

Bauer (1999) analyzed six commercial preparations (one to four batches each) containing *E. purpurea* (aboveground parts) expressed juice, and found that they varied dramatically in cichoric acid and alkamide content. HPLC analysis showed that the content of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobtylamide in the preparation ranged from 0.08 to 1.84 mg/100ml and cichoric acid varied from 0.0% to 0.4%.

In a recent issue of *Consumer Reports*, 12 brands of *Echinacea* pills on the U.S. market were compared. Levels of phenolic compound (caffeoyl-tartaric acid, chlorogenic acid, cichoric acid, and echinacoside) were assessed. Results showed that the average total percentage of phenolics varied from 0.8% to 4.5% depending on brand. Even within a brand, pills in different bottles had different levels of phenolics (Weil, 1999).

The independent ConsumerLab.com based in White Plains, New York, recently reviewed 25 commercial *Echinacea* products sold in the U.S., and tested them for the quality and quantity of *Echinacea* and levels of microbial contamination (ConsumerLab.com, 2001). Only 14 products (56%) passed this review. Others had inadequate labeling or lower levels of components than claimed on labels.

For 25 commercial *Echinacea*-containing remedies, Osowski et al. (2000) quantified cichoric acid and alkamide contents. Results showed large differences (up to 10,000-fold) in cichoric acid (Gilroy et al., 2003) or alkamide contents. Moreover, large differences among comparable products of different manufacturers and among different lots of the same product were noted.

This variation is caused in part by the enzymatic degradation by polyphenol oxidase (PPO; EC 1.14.18.1) during the processing of fresh plant material (Kreis et al., 2000). Enzymatic degradation during extraction could reduce the measured levels of phenolic compounds by more than 50%. Nusslein et al. (2000) have investigated the causes of cichoric acid degradation in *Echinacea* products and recommended a process to stabilize *E. purpurea* products**.**

Clinical Trials

Because of great diversity in *Echinacea* product quality, it is no wonder that the results of clinical trials are inconsistent. A number of clinical trials and reviews have indicated that *Echinacea* preparations are efficacious in preventing and treating the common cold and other respiratory infections, while other clinical trials (Barrett et al., 2002; Del Mar et al., 2002; Grimm and Muller, 1999; Turner et al., 2000) showed no significant effects. There are also a number of unpublished trials of *Echinacea* preparations with negative results (Melchart et al., 2001). In spite of this inconsistency, clinical studies of the effect of *Echinacea* on the common cold remain a valid subject (Turner, 2002); over 40 clinical studies have been published so far.

Schulten et al. (2001) reported a placebo-controlled, randomized, double-blind clinical trial evaluating the efficacy of the pressed juice from the fresh flowering *E. purpurea* in 80 patients with

the common cold. The results showed that the duration of all symptoms was significantly reduced (9.0 days to 6.0 days) and the disease was less severe in the active treatment group than in the placebo group.

In a study by Brinkeborn et al. (1999), acute treatments of the common cold with two tablets containing crude extracts of *E. purpurea* (95% herb, 5% root) three times daily were shown to significantly reduce cold symptoms compared to the placebo, while a preparation of *E. purpurea* root did not.

Another study on the prophylactic efficacy of *Echinacea* was carried out in the flu season with 647 students from the University of Cologne. The result showed a 15% reduction in the number of colds in the group given *Echinacea* compared to the placebo group (Winslow and Kroll, 1998).

In a 12-week, double-blind, placebo-controlled trial, 302 healthy volunteers were given *E. purpurea* or *E. angustifolia* root extracts or a placebo pill, and any effect on prevention of upper respiratory infections was noted. Subjects taking *Echinacea* lasted slightly longer before suffering infection and had slightly fewer colds than those given the placebo, but the differences were not significant (Melchart et al., 1998). Sixteen controlled clinical trials (involving 3,396 patients) from a total of 40 trials were chosen and evaluated in a Cochrane Library systematic review (Melchart et al., 2001). Most trials showed positive results, suggesting that *Echinacea* products may have some beneficial effects on prevention and treatment of the common cold. However, quality data in about two-thirds of the trials was considered insufficient. The biggest problem is the great diversity and the unclear comparability of the investigated products. The use of different *Echinacea* preparations made comparability of the results difficult. It was recommended (Dennehy, 2001; Osowski et al., 2000) that preclinical and clinical studies with *Echinacea*-containing herbal medicines should always indicate the species and plant parts used, formulation, method of extraction and quantification of potentially active components, and so on. These procedures will help to reduce inconsistencies in clinical trials and allow future research to focus on preparations that appear most promising.

Legislation, Pharmacopoeias, and Monographs

Considerable diversity also exists in the legislation, pharmacopoeias, and monographs of various countries. "There is no international consensus on how to regulate natural health products. The U.S. lists them as dietary supplements, with the onus on manufacturers to have data supporting their claims. At the other extreme, Germany regulates the products as drugs" (Sibbald, 2001). At present, in many countries, *Echinacea* is considered to be a food supplement, not a drug. Even so, as one of the most important herbal medicines in Western countries, *Echinacea* has been listed in some monographs as shown in Table 9.11.

In the U.S., *Echinacea* is classified as a dietary supplement according to the Dietary Supplement and Health Education Act (DSHEA) approved in 1994 (FDA, 1995). Dietary supplements are treated as foods by the FDA; if they were on the market before 1994, they did not have to undergo any evaluation (Roll, 2002). Therefore, it is a manufacturer's responsibility to ensure that *Echinacea* products are safe and properly labeled prior to marketing. This complicates verifying product purity, safety, and consistency. In 2001, the U.S. Pharmacopoeia (USP, 2002) created the Dietary Supplement Verification Program (DSVP) to help inform and safeguard the growing number of consumers who use dietary supplements. The program responds to the need to assure the public that dietary supplement products contain the ingredients stated on the product label (Thompson, 2001). As of March/April 2002, the botanical list in the U.S. Pharmacopoeia and National Formulary (USP-NF) had not yet included an official monograph of *Echinacea* products (DSVP, 2002).

In Canada, herbal products are divided into four groups under current Canadian Food and Drug regulation. *Echinacea* products are in the third group, which are nonprescription, traditional herb

TABLE 9.11
Regulation of *Echinacea* species and Parts by Various Organizations

Organization or Country	*E. purpurea*	*E. pallida*	*E. angustifolia*
World Health Organization (1999)	Aerial parts	Roots	Roots
European Scientific Cooperative on Phytotherapy (1999)	Aerial parts, roots	Roots	
German Commission E	Aerial parts	Roots	
British Herbal Pharmacopoeia (1996)			Roots
U.S. Pharmacopeia (2002)	Roots, leaves, flower, herbal powdered, and powdered extract	Roots, herbal powdered, and powdered extract	Roots, herbal powdered, and powdered extract
Canada	Roots		
Australia	Aerial parts, roots		Roots

Source: Data from World Health Organization, 1999, *WHO Monographs on Selected Medicinal Plants*, vol. 1, WHO, Geneva, pp. 136–144.

medicines (THM), intended for the self-treatment of a self-diagnosed, self-limiting condition (e.g., the use of *Echinacea* for the relief of sore throats due to colds). According to the Health Canada Drugs Directorate Guideline for THM such as *Echinacea* products (aqueous infusions and/or decoctions prepared from the dried root of *E. purpurea*), manufacturers follow Good Manufacturing Practice (GMP), provide a complete quantitative listing of ingredients on the label, indicate that a given product is a THM, and supply a minimum of two traditional references to support its pharmacological action for the part of the plant used (Thadani, 2002).

In Australia, complementary medicine may be either "listable" or "registrable" in the Australian Register of Therapeutic Goods (ARTG). *Echinacea* products are listable complementary medicines that may contain only substances generally regarded as safe, and may carry only claims for the temporary relief of minor self-limiting conditions. *Echinacea* products are thus available in pharmacies and health food stores for consumer self-selection (Cameron, 1998). Regarding *Echinacea* products, Australian Therapeutic Goods Administration (TGA) guides claim: "*Echinacea* helps support the immune system especially during the winter cold and flu season. This herb has been used traditionally for hundreds of years and now scientific evidence suggests that it may assist in supporting immune function" (TGA, 2001).

Vlietinck (2002) reported a European perspective on the elaboration of monographs on herbal medicinal products listed in the fourth edition of the European Pharmacopoeia. As of 2002, there were 106 published monographs, none of which focused on *Echinacea*; *E. angustifolia* radix, *E. pallida* radix, *E. purpurea* herba, and *E. purpurea* radix were among 40 monographs under study.

In Germany, Commission E of the Federal Institute of Pharmaceutical and Medicinal Products has not approved *E. angustifolia* herb and roots, *E. pallida* herb, or *E. purpurea* root for nonprescription drug use. Only research results from the fresh-pressed juice of *E. purpurea* flowering herb in 22% ethanol by volume as a preservative and from the water-alcohol extract of *E. pallida* roots qualified for an approved monograph (Blumenthal et al., 2000). The latter preparations are recommended as a supportive treatment of recurring infections in the bronchial area and urinary tract, as well as for external use in the case of poorly healing superficial wounds (Bauer, 1999).

Echinacea products are sold as food supplements in Norway, but as herbal medicines in Sweden, Finland, and Denmark, and must be registered.

QUALITY STANDARDIZATION OF *ECHINACEA*

Although *Echinacea* products belong to the top best-selling group of herbal products, thus far its cultivation, harvesting, and extraction are realized without profound knowledge of factors that affect its quality. Commercially available preparations of varying quality are the result.

The increasing popularity of *Echinacea* has raised concerns in the herbal medicine community and the media that there is a need to establish standards for *Echinacea* products. The diversity described above supports the need for greater efforts to provide authentic, safe, stable, and efficacious *Echinacea* products that are consistent from batch to batch (Bauer, 1999; Grant and Benda, 1999).

Standard quality controls with scientific criteria start with a defined species, proper cultivation and harvesting through a defined drying and extraction procedure, and end with a quantitative determination by a defined method for one or more of its active ingredients (Tierra, 1999).

Active Markers

In order to standardize *Echinacea* preparations, some suitable active markers must be identified in the products. Although a number of active components have been studied and identified, their mechanisms of action and bioavailability are not yet completely understood (Barrett, 2003). At present, alkamides and cichoric acid content seem to be used as quality markers for some *Echinacea* products. However, it is noteworthy that echinacoside, which is used frequently for standardizing *E. pallida* and *E. angustifolia* extracts, is absent from *E. purpurea* (Table 9.5). Therefore, depending on the plant species used, the active marker should be appropriate. And since the active components may act additively or synergistically, the overrating of a single compound in quality control should be avoided (Bauer, 1999).

As mentioned above, active marker levels depend on growing conditions, climate, soil quality, and harvest time, and all factors in the processing stage. Variation in the commercial samples and manufacturing process can be qualitatively and quantitatively revealed by various improved chromatographic methods that have been used to measure content levels of typical components in the plants and products of *Echinacea* species (Pomponio et al., 2002).

Alkamides and Cichoric acid

Bauer (1999) described an HPLC method for identifying alkamides and cichoric acid in commercial samples of *E. purpurea* pressed juice preparations, and proposed standardization by analyzing alkamide and cichoric acid contents. These components are typically found in *E. purpurea* and show pharmacological activity. However, in the study of Al-Hassan et al. (2000), cichoric acid was not found in the pressed juice.

Alkamides have phagocyte-stimulating activity *in vitro* and *in vivo*. They were also shown in some cases to inhibit enzymes 5-lipoxygenase and cyclooxygenase, which are involved in inflammation. Cichoric acid inhibits hyaluronidase and causes stimulation of phagocyte activity *in vitro* and *in vivo* (Bauer, 1999; Clifford et al., 2002). It is also an antioxidant protecting against free radical–induced injury (Hu and Kitts, 2000; Sloley et al., 2001) and has also been shown to selectively inhibit human immunodeficiency virus type 1 integrase (McDougall et al., 1998). However, a more recent animal study (Goel et al., 2002) indicated that purified cichoric acid and the polysaccharide component from *E. purpurea* failed to exert any immunostimulatory effects in rats. These authors provided *in vivo* evidence that only the lipophilic alkamides (dodecatetraenoic acid isobutylamides) are the effective, nonspecific immunomodulatory agent in *Echinacea* plant extract. Alkamides appear to be the most active agents in terms of stimulating effect on the alveolar macrophage function (stimulating effects on TNF-α and nitric oxide production) in normal rats.

Echinacoside

Echinacoside is a polyphenolic caffeoyl derivative with antioxidant activity. It seems that the anti-inflammatory activity of *E. pallida* root extract depends on the presence of echinacoside. Recent studies on antiinflammatory and cicatrizing activity of Italian-grown *E. pallida* root extract extracted by ethanol (1:10 w/v) (Speroni et al., 2002) proved that rats treated with echinacoside or dried *E. pallida* extract showed significantly higher antiinflammatory and wound-healing responses than did the control or *E. purpurea* group. Hu and Kitts (2000) found that the methanolic extract of *E. pallida* root exhibited greater antioxidant activity than extracts of *E. angustifolia* or *E. purpurea*. Therefore, echinacoside could be used as the active marker of *E. pallida* species and its products.

Polysaccharides

Two polysaccharides (PS I, PS II) have been isolated in the aerial parts of *E. purpurea* (Bauer and Wagner, 1991), and *Echinacea*-derived polysaccharides are indeed active in certain immunological models (Barrett, 2003; Emmendörffer et al., 1999). Bodinet and Beuscher (1991) reported that the roots of *E. purpurea* contain arabinogalactans and arabinogalactan-containing glycoproteins that exert immunomodulating activity. They indicated that the glycoprotein-containing fractions of *E. purpurea* root extracts are able to induce the secretion of TNF-α, IL-1, and INF-α and -β. Burger et al. (1997) also showed that the polysaccharide component of *Echinacea* has the effect of increasing *in vitro* production of TNF-α, IL-1, and IL-6 by macrophage.

More results for *Echinacea* polysaccharides are not from plant sources but rather from cell cultures of *Echinacea.* Polysaccharide components from plant sources were structurally different compared to those obtained from cell cultures. *Echinacea* preparations commonly contain pharmacologically insignificant amounts of polysaccharides (Bone, 1997a). Therefore, more research should be undertaken if polysaccharides are to be used as an active marker for *Echinacea* products.

STANDARDIZATION

Standardization has become a major trend in the herbal products industry as well as research organizations (Roll, 2002; Tierra, 1999; Vlietinck, 2002). The first step in conducting quality control of *Echinacea* products is to establish true botanical identity and safety of its raw plant material by comparison with authenticated reference plant material (ARPM), which aids in the identification of adulterants (Roll, 2002). It is expected that a "certificate of botanical identity" will eventually be required for all sales of *Echinacea*. For standardization of *Echinacea* cultivation, especially for determining optimum harvest time, practice-relevant results are needed, including botanical characteristics (macroscopic and microscopic) (Giancaspro, 2000), gross physical determinants of quality, and widely accepted quality criteria relating to chemical content. Strict quality controls are required for the *Echinacea* plant raw materials with regard to homogeneity and purity of the raw material, minimum content of effective components, and limit values for plant-protective agent residues and microbial contamination.

A number of quantitative standards for *Echinacea* roots already exist. The USP typically specifies maximum water content of 10%, total ash content of up to 7%, not more than 3% of foreign organic matter, not more than 4% of acid-soluble ash, and not more than 0.001% of heavy metals (Giancaspro, 2000). The chemical identification of *Echinacea* roots has been intensively studied and could be performed by a specific procedure of the TLC test (Giancaspro, 2000) (Table 9.12) and HPLC analyses (Perry et al., 2001).

Numerous tests can be used to evaluate the quality and purity of end products in *Echinacea*. First, the physical characteristics of the extract, including appearance, pH, solubility, content of

TABLE 9.12
Identification of *Echinacea* Roots and Powdered Extracts via Thin Layer Chromatography Procedure

Constituents	*E. purpurea*	*E. pallida*	*E. angustifolia*
Echinacoside	+ Yellowish (365 nm)	+ Yellowish (365 nm)	— or trace
Cynarine	+ Yellowish (365 nm)	—	—
Alkamides	+ Blue black (254 nm)	—	+ Blue black (254nm)
Cichoric acid	—	—	+ Yellowish-green (365nm)
Caftaric acid	—	—	+ Yellowish-green (365nm)
Ketoalkenyne	—	+ Green-brown-violet	—

Source: Data from Giancaspro, G., 2000, *Pharmacopeial Forum,* 26: 1578–1596.
—, no spots seen on TLC plates.

total solids, ash content, and in the case of dried extracts, particle size, may be examined. Next, appropriate quantities of desired ingredients contained in the extract may be analyzed. Chromatographic (TLC, HPLC, GC) and spectroscopic (UV, IR) analysis may be used for this (Bauer, 1999; Bauer and Remiger, 1989; Bergeron et al., 2000; Gilroy et al., 2003; Schieffer and Kohn, 2002; Wagner, 1996). Finally, the extract may be tested for impurities such as residual solvents, herbicides, pesticides, and microbial contamination (Center for Food Safety and Applied Nutrition, FDA, 1999; Roll, 2002). The requirements of microbiological tests of *E. purpurea* products are listed in Table 9.13 (WHO, 1999). Mycotoxins and radioactivity should be absent.

Since the active ingredients in *Echinacea* are complex and not yet completely known, the quality of *Echinacea* extracts is assessed by a "fingerprint" chromatogram. The "fingerprint" chromatograms of alkamides and phenolic derivatives in the root extracts of *E. angustifolia*, *E. pallida*, and *E. purpurea* provide enough information to reach a reasonable conclusion about quality and could be used for comparative and relative quality assessment of *Echinacea* samples. A standard procedure of liquid chromatography for the analysis of total phenols and alkamides in the roots and their extracts can be found in the USP (Giancaspro, 2000). The typical retention times for caftaric acid, chlorogenic acid, echinacoside, cichoric acid, and cynarine are about 6.8, 7.2, 10.3, 16.4, and 17.5 minutes, respectively, detected at 330 nm, performed with the Prodigy ODS-3, 100Å, 15.5% of carbon load, and end capped of 5-µm L1 column. The typical retention times for dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide are 20.0 and 21.0 minutes, respectively, detected at 254 nm, performed with the Luna C18 (2) brand of 5-µm L1 column. Table 9.14 lists the chemical criteria of *Echinacea* roots and their powdered extract (extraction ratio 2:1 to 8:1) in USP (Giancaspro, 2000). It should be mentioned that the lack of commercially available reference standards of active principles presents a problem in establishing quantitative information by chromatography.

Some extracts are labeled and sold as standardized extracts. For example, echinacoside is the desired compound present in some *Echinacea* extracts (*E. angustifolia* or *E. pallida*). A capsule containing 250 mg of *Echinacea* extract standardized to 4% would contain 10 mg of echinacosides (Center for Food Safety and Applied Nutrition, FDA, 1999). As a standard *Echinacea* product, its label should indicate the related information to consumers. However, at present most *Echinacea* product labels give little information about which species of *Echinacea* was used and the amount of the active ingredient in the *Echinacea* remedy (Gilroy et al., 2003).

Since March 1999, the FDA has required that herbal products like *Echinacea* should provide labels identifying the species of the herb, the part of the plant used, and the concentration of the herb. ConsumerLab.com in the United States has published a review on testing *Echinacea* products

TABLE 9.13
Microbiological Testing Requirements for *Echinacea purpurea* Products

Preparations	Microbes (/g or ml)				
	Aerobic Bacteria	Fungi	*E. coli*	*Salmonella* sp.	Enterobacter and Some Gram-negative Bacteria
Decoction	$< 10^7$	$< 10^5$	$< 10^2$	0	0
Internal use	$< 10^5$	$< 10^4$	0	0	$< 10^3$
External use	$< 10^2$	$< 10^2$	0	0	$< 10^1$

TABLE 9.14
Chemical Criteria of *Echinacea* Roots and Powdered Root Extract

Chemical Components	*E. angustifolia*		*E. pallida*		*E. purpurea*	
	Roots	Extract	Roots	Extract	Roots	Extract
Total phenols	> 0.5%[a]	> 4% < 5%[a]	> 0.5%[b]	> 4% < 5%[b]	> 0.5%[b]	> 4%[b]
Dodecatetraenoic acid isobutylamides	> 0.075%	> 0.6%			> 0.025%	> 0.025%

[a] Calculated on the dried basis as the sum of caftaric acid, cichoric acid, chlorogenic acid, echinacoside, and cynarine.
[b] Calculated on the dried basis as the sum of caftaric acid, cichoric acid, chlorogenic acid, and echinacoside.
Source: Data from Giancaspro, G., 2000, *Pharmacopeial Forum,* 26: 1578–1596.

(ConsumerLab.com, 2001). According to ConsumerLab.com, *Echinacea* product labels should meet the following requirements:

1. Provide all of the following information on labels (as required by the FDA):
   a. The species of *Echinacea* (i.e., *E. purpurea*, *E. angustifolia*, or *E. pallida*)
   b. The part of the plant used, such as root or aerial (aboveground or also referred to as the "herb") portions (including stem, leaves, and flowers)
   c. The form (whole herb or root, extract, or tincture)
   d. The amount of *Echinacea* per pill or dose in grams (g) or milligrams (mg)
2. Products labeled as containing the roots of *E. angustifolia* and/or *E. pallida* are required to contain detectable levels of the specific marker compound echinacoside; products labeled as containing roots or herb of *E. purpurea* are required to contain detectable levels of cichoric, caftaric, and chlorogenic acids, but if they were *E. purpurea*–only products, they should not have more than trace levels of echinacoside.

However, even when the label indicates the chemical standard used, potency can still vary considerably. On the one hand, this is because the pharmacological activity of *Echinacea* may also involve the combined or synergistic actions of various compounds (Ang-Lee et al., 2001). On the other hand, as Gilroy et al. (2003) indicated, *Echinacea* samples labeled as "standardized" did not guarantee that the samples contained as much as was stated on the label. They found after an investigation of 59 commercial samples that actual contents matched contents listed on the label in only 52% of the samples. Of the 21 "standardized" preparations, 43% met the quality standard described on the label, and only four (7%) of the samples met the FDA's labeling requirements. Clearly, a lot of scientific work aimed at the crucial task — quality control and standardization of *Echinacea* preparations — remains to be carried out.

CONCLUSION

Studies focusing on identifying active constituents, elucidating their mechanisms of action, investigating various factors that cause differences in product quality, and finally, establishing the scientific standardization of *Echinacea* preparations have already contributed much. Yet more is still necessary to fully define this clinically promising herbal product in order to provide more definitive evidence for its medicinal use.

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Section V

In Vivo *and* in Vitro *Experimental Evidence for the Immuno-Enhancing Activity of* Echinacea *Species*

10 *Echinacea in Vivo*: A Prophylactic Agent in Normal Mice and a Therapeutic Agent in Leukemic Mice

Sandra C. Miller

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INTRODUCTION

Although herbal medicine was practiced by U.S. physicians in the 19th and early 20th centuries, *Echinacea* was never approved by the American Medical Association because rigorous experimental evidence of its medical efficacy did not exist, and in fact, the healing properties of this herb were virtually forgotten with the development of antibiotics (Combest and Nemecz, 1997). Subsequently, however, techniques for measuring the functional response of different immune cells, at least *in vitro*, led to herbs such as *Echinacea* being rediscovered and immune stimulation was advanced as a possible mechanism for their medicinal value. During the past 2 decades, much effort has been devoted to analyzing the many chemical compounds from this plant that may act on specific immune cells. These studies have indicated that such compounds include high molecular weight polysaccharides, inulin, heteroxylan, essential oils, alkyamides such as echinacein, isobutylamides (pentadecadienes and hexadecadienes), polyacetylene, tannins, vitamin C, and flavonoids. From this list, some important immunoenhancing elements may be those that interfere with prostaglandin formation, since prostaglandins are detrimental to natural killer (NK) cells. NK cells are fundamental as the first line of defense against a host of invading pathogens. We found some years ago that *in vivo* administration of an *inhibitor* of prostaglandin (i.e., indomethacin) significantly *increased* NK cells in leukemic mice, concomitant with cure and/or significantly longer life span (Christopher et al., 1991; Dussault and Miller, 1993). In the same way, the alkamide family of compounds within *Echinacea* inhibits the production of 5-lipoxygenase and cyclooxygenase (Muller-Jakic, 1994; Wagner et al., 1989), key enzymes in the production of prostaglandins, leading, thus, to an increase in the NK cell population, by reducing/removing the negative agent, prostaglandin. Thus, any treatment that would augment such cells would clearly be worthy of investigation for its therapeutic/prophylactic potential.

NK cells, unfortunately, decline with age, and correspondingly, several types of cancer increase with age in both mice and humans. This relationship is undoubtedly more than coincidental. Some years ago, we established the mechanism for the age-related decline in NK cells (Dussault and Miller, 1994) and found that it results from a least two phenomena: (1) reduced new cell production in the NK cell lineage in the bone marrow birth site, and (2) reduced efficiency of mature NK cells to bind to their target cells, hence preventing subsequent killing of the offensive target, such as virus-infected or cancer cells. Moreover, a growing body of anecdotal and experimental evidence suggested that certain phytochemicals in herbs might have the capacity to reduce tumors and virus infections (Bauer, 1996; Melchart et al., 1995; See et al., 1997). Considerable evidence had accumulated indicating the presence of immunostimulating compounds within *Echinacea* (Bauer, 1996; Muller-Jakic et al., 1994; Roesler et al., 1991a, 1991b; Steinmuller et al., 1993). One such compound is the complex carbohydrate, arabinogalactan. Macrophages, fundamentally important "helper" cells for the functional activity of NK cells, release numerous cytokines upon stimulation with purified polysaccharides such as and including arabinogalactan (Bauer, 1996; Leuttig et al., 1989; Stimpel et al., 1984). Among the resulting cytokine cascade produced by such stimulated macrophages are several powerful NK enhancers, such as interferon and TNF-α (Hauer and Anderer, 1993; Kelly, 1999; Leuttig et al., 1989; Rininger et al., 2000; Stein et al., 1999).

Thus, all these studies have collectively shown that while the polysaccharide, arabinogalactan, results in the production of NK *stimulators*, other *Echinacea*-derived phytochemicals (i.e., the alkamides) can release NK cells from their natural endogenous *inhibitors*, the prostaglandins. Consequently, a combination of all the positive data (anecdotal and experimental), emerging from the results of *in vivo* administration of *Echinacea* (Hill et al., 1996; Lersch et al., 1990, 1992; Roesler et al., 1991a, 1991b; Steinmuller et al., 1993; Stimpel et al., 1984; Tragni et al., 1985), led to our hypothesis that administration of *Echinacea* to leukemic mice may lead to the reduction and/or cure of these retrovirus-mediated cancers. Furthermore, we hypothesized that therapeutic intervention with *Echinacea* as an NK cell enhancer in combination therapy could be very successful in leukemia treatment. Antitumor immunotherapy, whereby immunization is combined with some pharmaceutical or secondary treatment, is coming into use clinically, and is in under considerable experimental testing.

Of fundamental importance for the use of any agent, either prophylactically or therapeutically, especially over the long term, is that it not be, by itself, as deleterious (toxic) to the host as the disease(s) for which it is administered. In the case of *Echinacea* species, there is considerable evidence that, indeed, there appears to be no overdose/toxicity level as defined by assorted criteria (Lersch et al., 1992; Melchart et al., 1995; Mengs et al., 1991). Consequently, in our own studies, we chose a dose that was at the top of a dose–response curve prior to its plateau, as measured by progressive increases in the absolute numbers of NK cells. No further increase in NK cell numbers was found using a dose beyond 0.45mg/25g body weight per day, at least for the specific brand of *E. purpurea* employed throughout the studies discussed below.

EVIDENCE OF A PROPHYLACTIC ROLE FOR *ECHINACEA*

We undertook a study a few years ago (Sun et al., 1999) to investigate the changes in immune system cells — as well as other hemopoietic cells — that may result from dietary intake of *Echinacea.* We added to the daily diet of inbred mice, for either 1 week or 2 weeks, *E. purpurea* extract from a commercial supplier (Phyto Adrien Gagnon, Santé Naturelle (A.G.) Ltée, La Prairie, QC, Canada), whose product is readily available in the marketplace and consumed by the general public. Thus, under controlled laboratory conditions, we analyzed the hemopoietic and immune cell populations in the spleen and bone marrow of normal, young adult mice, with and without *E. purpurea* in their daily diet for 1 week or 2 weeks. The spleen is a vast repository for cells mediating

specific immunity (T and B lymphocytes), as well as nonspecific immunity (NK cells, monocytes/macrophages) and other cells involved in the generalized disease defense process (mature granulocytes). The bone marrow is the birth site of all abovementioned cells, and hence a repository of the precursor cells for all these lineages.

Our results indicated that mice fed *E. purpurea* daily for either 1 week or 2 weeks, had, in absolute numbers, significantly more NK cells (identified by immunoperoxidase labeling methods) in their bone marrow than did the bone marrow of mice consuming untreated chow ($p < 0.01$). The spleen (to which bone marrow-derived, new NK cells travel almost exclusively) had approximately 25% more NK cells in mice fed *E. purpurea* for 1 week, and significantly more NK cells ($p < 0.01$) after 2 weeks of daily dietary consumption of the herb. Moreover, monocytes/macrophages, accessory cells for NK cells, were approximately 25% more plentiful in both the bone marrow and spleen of mice consuming *E. purpurea* for 1 week, and were significantly more numerous in the spleen ($p < 0.01$) and bone marrow ($p < 0.0l$) of mice consuming the herb for 2 weeks. Especially important is the fact that increased NK cells in the bone marrow necessarily means that these new NK cells had been produced there under the influence of the dietary *Echinacea*, since NK cells do not recirculate back to the bone marrow once they exit that organ (Miller, 1982; Seaman et al., 1978; Zoller et al., 1982). In other words, increased NK cells in the bone marrow necessarily resulted from increased production of these cells, under the influence of *E. purpurea*. Strikingly, moreover, all other lymphocyte populations, as well as the mature granulocytes, granulocyte precursors, and red blood cell precursors, remained steadfastly at control (untreated chow) levels in both the spleen and the bone marrow, whether mice were fed *E. purpurea* for 1 week or 2 weeks. Therefore, this study, incorporating the parameters of herb exposure time, host animal pedigree, age, health, gender, and living environment, demonstrated singularly positive influences of *E. purpurea* on NK cells and their accessory cells, the monocytes/macrophages. This study represents the first quantitative *in vivo* analysis demonstrating the effects of *Echinacea* on the hemopoietic and immune cell populations in the organs of their birth (bone marrow) and function (spleen) under controlled laboratory conditions. The fact that these results were found in normal, healthy young adult animals indicates a potentially prophylactic role for *E. purpurea.*

ECHINACEA CAN REJUVENATE NK CELLS IN ELDERLY ANIMALS

The observations of our study above prompted a systematic investigation of the potential NK-stimulating role of *E. purpurea* in aging mice under the same conditions. Furthermore, since we had now demonstrated that NK cell production is augmented in the bone marrow in young adult mice in the presence of *E. purpurea*, we hypothesized that this may also occur in elderly mice, the latter group normally exhibiting little or no new NK cell production (Albright and Albright, 1983; Dussault and Miller, 1994; Ghoneum et al., 1991; Hanna, 1985; Krishnaraj, 1992; Kutza and Murasko, 1994). Consequently, we completed a study recently (Currier and Miller, 2000) which demonstrated that in healthy elderly mice, it was possible not only to increase NK cell numbers but their function as well by adding *Echinacea purpurea* to the daily diet of normal elderly mice for only 2 weeks. Both parameters (NK cell numbers and function) are diminished, or very reduced, in normal elderly humans as well as elderly mice. Indeed, this herbal addition to the diet of elderly mice returned their NK cell numbers and function to the levels of their young adult counterparts. In elderly humans, exogenous administration of various cytokines and growth factors results in little or no stimulatory influence on a variety of immune parameters (Kawakami and Bloom, 1988; Kutza and Murasko, 1994; Lerner et al., 1989). Similarly, we had previously found in healthy elderly mice that neither the cytokine, IL-2, nor the pharmaceutical agent, indomethacin (both potent stimulators of NK cells in the young adult animal), was able to stimulate its NK cell numbers or function (Dussault and Miller, 1994). Specifically, we found that giving this herb via the chow

to old mice every day for 2 weeks resulted in an increase in absolute number of NK cells in the bone marrow, from almost undetectable numbers to significantly increased numbers ($p < 0.004$), equivalent to levels seen in young adult bone marrow. These results clearly indicate that this herb has been able to actually stimulate new NK cell production in the aged mice, after NK cells had undergone the natural age-related decline. Moreover, in the spleen, which is by far the major recipient organ for virtually all bone marrow–derived NK cells (Miller, 1982), the absolute numbers of NK cells were 30% greater than in control mice consuming untreated chow. However, no positive influence was found on the absolute numbers of the mature or precursor granulocytes, precursors to red blood cells, or immune cell (lymphocytic) populations after 2 weeks of ingesting *E. purpurea*, in either the spleen or the bone marrow in accordance with our previous observations in young adult mice (Sun et al., 1999).

Our study (Currier and Miller, 2000) also demonstrated that the actual lytic capacity, that is, ability to kill tumor cells, of this newly produced army of NK cells in these elderly mice was also returned to levels equal to those of young adults. In other words, we found that there was a consistent and statistically significant elevation in tumor killing (cytolytic) activity ($p < 0.03$ to 0.001) by NK cells taken from healthy aged mice that had been fed *Echinacea* for 2 weeks versus those fed regular untreated chow.

This study was especially pivotal since it demonstrated that the herb *E. purpurea* had the capacity to rejuvenate NK cells, a major element in the disease defense armament, in terms of both numbers and function. This rejuvenation ability could not be achieved by other NK-cell stimulants that were so successful in young adults.

ARABINOGALACTAN AUGMENTS NK CELLS

In a recent study (Currier et al., 2002), we injected arabinogalactan intraperitoneally into young adult and elderly inbred mice once daily for either 1 week or 2 weeks. The specific arabinogalactan used is a water-soluble, complex carbohydrate form (L-arabino-D-galactans), a highly branched molecule with branched backbone chains of (1-3/6)-linked β-D-galactopyranosyl residues to which are attached side chains containing L-arabinofuranosyl, L-arabinopyranosyl residues. In striking contrast to our observations of increased NK cell numbers 1 week after daily administration of whole *Echinacea* (Sun et al., 1999), the results of administering arabinogalactan alone to healthy young adult mice for 1 week significantly *decreased* NK cell numbers in the bone marrow ($p < 0.02$), and resulted in no change from control numbers in the spleen (Currier et al., 2002). However, by 2 weeks after daily exposure to arabinogalactan, NK cell numbers in the bone marrow had risen to control levels and in the spleen they were significantly increased ($p < 0.004$), almost double the control numbers. Thus, unlike whole *Echinacea*, the effects of which were readily evident as stimulation of new NK cell production in the bone marrow by 1 week (Sun et al., 1999), it appeared that 2 weeks were needed to produce any stimulatory effect on NK cells when the polysaccharide alone was employed. Moreover, that observation appeared to be the only positive effect of this polysaccharide in these healthy young adult animals. The lymphocytes (T, B cells) were significantly decreased after 1 week ($p < 0.004$) and 2 weeks ($p < 0.001$) of arabinogalactan administration in bone marrow. With respect to the other hemopoietic cell lineages, arabinogalactan had no influence on them after 1 week, but after 2 weeks, in the spleen, mature granulocyte numbers, as well as their precursors and cells of the monocyte/macrophage lineage, were significantly reduced ($p < 0.006$, $p < 0.043$, and $p < 0.001$, respectively), while remaining unchanged in the bone marrow (Currier et al., 2002).

In striking contrast to our observations on elderly mice given whole *Echinacea* (Currier and Miller, 2000), administration of arabinogalactan alone for 2 weeks was completely ineffective in augmenting NK cells in either the bone marrow or spleen, and was similarly ineffective in augmenting other non-NK lymphocytes (Currier et al., 2002). This analysis has demonstrated that

although a single phytocompound, in this case, a complex carbohydrate of the type contained in *Echinacea* species, is capable of enhancing NK cells, the time taken to do so is longer (2 weeks) and, moreover, there is by this time a negative influence on other important disease-defense cell lineages (granulocytes, monocyte/macrophages). Furthermore, it appears that arabinogalactan administered to normal elderly mice is incapable of stimulating NK cells in either the bone marrow or spleen, and has no influence on all other immune and hemopoietic cells in these organs.

Thus, in the long run, it may be more efficacious in terms of prophylaxis and/or therapy to administer whole *Echinacea* rather than isolated phytochemicals contained therein. Whole product contains multiple compounds, each serving either different or synergistically acting physiologically significant functions. The possibility that the collective whole may indeed be better than any single derivative is supported by circumstantial evidence provided by others (Rininger et al., 2000; Voaden et al., 1972).

ECHINACEA GIVEN TO LEUKEMIC MICE ENHANCES NK CELLS AND INCREASES LIFE SPAN

Before 2001, the literature contained no information concerning the status of immune cells and other hemopoietic cells in leukemic (or any tumor-bearing) animals or humans given therapy involving herbals or derived phytocompounds. We recently undertook a study to investigate the role of dietary *Echinacea* in leukemic mice (Currier and Miller, 2001). The study was completed under controlled laboratory conditions, including the use of (1) inbred mice of identical strain, age, and gender; (2) regulated dose and known exposure times of *E. purpurea*; (3) known stage of leukemia development; and (4) standardized housing conditions throughout the investigation for all treated and untreated (control) leukemic mice. Leukemias and lymphomas have long been known to be readily killed by NK cells (Biron and Welsh, 1982; Hefeneider et al., 1983; Itoh et al., 1982; Kalland, 1987; Kasai et al., 1981; Keissling et al., 1975; Koo and Manyak, 1986; Lotzova et al., 1986). Moreover, these tumors are virus associated, and virus-infected cells are prime targets for NK cells. We hypothesized, consequently, that any agent that enhances NK cells should be expected to be effective in leukemia abatement. Thus, *E. purpurea* was given via the daily diet from the day of tumor onset (instigated by injection of 3×10^6 live, FLV-induced leukemia cells) and concluding approximately 3 months later.

The results were strikingly positive. NK cell numbers 9 days after the onset of the leukemia were very significantly elevated over those of leukemic mice fed untreated chow ($p < 0.000007$). Three months after leukemia onset — long after *all* the leukemic mice fed untreated chow had died (27 days after tumor onset) — the absolute numbers of NK cells in the treated mice were recorded at more than twice the level found in *normal* mice of the same age. Moreover, an analysis of all the hemopoietic cell populations in the bone marrow of these leukemic mice at 3 months after leukemia onset revealed that the cell numbers in all major cell lineages were virtually indistinguishable from our previously established findings in normal mice. Thus, this fundamental study demonstrated first, that in the presence of dietary *E. purpurea,* resumption of normal hemopoiesis and lymphopoiesis in these leukemic mice had occurred (at 3 months), concomitant with the significant increase in the leukemia-fighting NK cells. Second, the life-span analysis revealed that approximately one-third of leukemic mice not only survived until 3 months, but went on to long-term survival and normal life span (Currier and Miller, 2001). The data, when analyzed by Kaplan-Meier Statistics software, revealed that the survival advantage provided by adding *E. purpurea* to the diet of leukemic mice compared to mice consuming the control diet was statistically significant ($p < 0.022$). Nevertheless, survival frequency could undoubtedly be improved even more by manipulation of dose/frequency/duration regimens of *E. purpurea* in the diet.

Thus, it is clear that phytocompounds contained in *E. purpurea*, and possibly other *Echinacea* species, may be profoundly valuable tools, at least in combating leukemia and likely in the

amelioration of other types of tumors yet untested. Clearly, the therapeutic potential of this herb suggests that it could have a formal and fundamental role to play in modern antitumor therapy, either alone or in combination protocols.

ECHINACEA IN COMBINATION THERAPY ENHANCES NK CELLS AND INCREASES LIFE SPAN OF LEUKEMIC MICE

In other experiments, we co-administered to leukemic, *E. purpurea*-consuming mice (as above), the pineal gland hormone melatonin from leukemia onset. This substance is a neuroimmunomodulator, a biogenic indoleamine (N-acetyl-5 methoxytryptamine), long known to be a chronomodulator in biologic systems and, more recently, identified as a powerful immunostimulant, specifically involving NK cells (Demas and Nelson, 1998; Guerrero and Reiter, 1992; Liebmann et al., 1997; Maestroni et al., 1996; Poon et al., 1994; Yu et al., 2000). We found (Currier and Miller, 2001) that the combination of melatonin and *E. purpurea* co-administered in the diet of leukemic, young adult mice increased the survival rate from the approximately 33% achieved by *E. purpurea* alone, to 40%, such that Kaplan-Meier statistical analysis of survival indicated significance at $p < 0.00035$ when the two agents were administered together versus that found by giving *E. purpurea* alone ($p < 0.022$). Thus, at least in leukemic animals, adding a second NK stimulant (melatonin) proved to be more efficacious than therapy employing *E. purpurea* alone.

In a sequel to the study above, we assessed the effect of combination therapy using immunization with killed leukemia cells prior to the onset of leukemia, followed by dietary administration of *E. purpurea* (Currier and Miller, 2002). Studies involving tumor immunization have employed a wide variety of protocols, including genetic engineering of tumor cells with and without viral modification or injecting killed tumor cells or their extract (Carr-Brendel et al., 1999; Charles et al., 2000; Li et al., 1998, Okamoto et al., 2000; Schirrmacher et al., 1998, 1999; Simons et al., 1999). We postulated that the combination of immunization against leukemia together with dietary *E. purpurea* could be substantially more therapeutic than either *E. purpurea* alone or immunization alone. Thus, inbred mice of identical strain, age, and gender were given killed leukemia cells 5 weeks before injecting them with 3×10^6 live leukemia cells to initiate tumor onset. The results indicated that immunization therapy *alone* produced a survival rate and life span increment similar to that provided by administering *E. purpurea alone*, that is, approximately one-third of the treated population survived long term (Currier and Miller, 2001, 2002). When *E. purpurea* was added to the diet from tumor onset to these immunized mice, the survival rate and life span increment nearly doubled to almost 60% (Currier and Miller, 2002). When NK cells were assessed at 3 months after tumor onset in these mice receiving combination therapy, it was found that the absolute numbers of NK cells in the bone marrow rose to almost three times that of immunized mice *not* consuming *E. purpurea* ($p < 0.003$), while the numbers of NK cells in the spleens of immunized mice consuming *E. purpurea* rose to almost twice ($p < 0.00l$) the levels of immunized mice that did not consume the herb. Moreover, by 3 months, the presence of *E. purpurea* in the diet had no influence on the lymphocytes (T, B cells), monocytes, mature granulocytes, or their precursors in either the spleen or the bone marrow, again demonstrating the primary and positive influence of *Echinacea* on NK cells.

These results indicate that combination therapy can have profoundly positive results, where one of the agents is *E. purpurea*, as long as the other agent is neither cytotoxic nor immunosuppressive. For example, agents such as cyclophosphamide, methotrexate, and a battery of other chemotherapy poisons that indiscriminately kill vast numbers of normal cells along with their tumor targets, must be excluded from any combination therapy with *E. purpurea* or other *Echinacea* species.

We have thus established under formal experimental conditions that using *Echinacea* alone, or even more effectively, in combination treatment with an appropriate secondary treatment, signifi-

cantly increases survival rate and life span, at least in mice, and would appear to warrant further investigation in larger mammals and humans. Both *E. purpurea* and melatonin are commercially available and ready options for leukemia-afflicted humans, especially where other forms of therapy have proven to be too toxic to endure, or have become ineffective.

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11 Effect of *Echinacea* on Cells Involved in Disease Defense

Helena Šestáková and Bohumil Turek

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INTRODUCTION

The plants of the genus *Echinacea* possess a number of known bioactive properties, including antioxidant and anticarcinogenic effects. The study of parts of plants of genus *Echinacea* or of their components, in terms of their capacity to influence the immune mechanisms of an organism, is therefore very important for the assessment of protection against various pathogens.

Immunologic studies are generally concerned with the response of an organism to foreign (extraneous) substances entering the body. The basic function of the immune system is to differentiate between "foreign" and "one's own" molecules, and to protect against extraneous proteins. An immune reaction takes the form either of a specific response mediated by T and B cells, or of a nonspecific (natural) response mediated by macrophages, natural killer cells, and polymorphonuclear leukocytes (PMNLs). A positive or negative effect on immunity of substances obtained from plants or of nutritional factors is therefore very important for maintaining the integrity of an organism. One of the most important mechanisms underlying the natural defense of an organism is phagocytosis. Quantitative as well as qualitative insufficiency of the system of phagocytosis results, among other things, in an increased sensitivity of an individual to infectious agents. PMNLs are responsible for natural defense, and actively emigrate from the circulation to the site of inflammation in response to a signal in the form of a chemotactic factor (Dahlgren, 1989; Schiffmann and Gallin, 1979; Wilkinson, 1983). In addition to affecting the mobility of phagocytes, chemotactic factors can trigger the oxidative metabolism of these cells, with subsequent formation of oxygen free radicals and the release of lysosomal enzymes (Badwey and Karnovsky, 1980; Dahlgren, 1989; Klebanoff, 1980). PMNLs are activated by various phagocytotic stimuli, including bacteria and allergens, and by carcinogenic substances (Klein et al., 1991). The activation of the PMNL membrane is followed by the so-called burst of oxidative metabolism (respiratory flare-up) usually associated with phagocytosis. Ligands binding to receptors in the cytoplasmic membranes of the phagocytes disturb their structure, activating NADPH oxidases. These oxidases catalyze electron transport from NADPH to oxygen, reducing it to a superoxide radical. This, in turn, is reduced to hydrogen peroxide either spontaneously or through catalysis by superoxide dismutase. The superoxide anion gives rise not only to hydrogen peroxide but also to other cytotoxic forms of oxygen. These forms of oxygen are not dependent on the fusion of a phagosome with a lysosome in the phagocyte. However, once this fusion occurs, the enzyme myeloperoxidase can enter the

phagolysosome, forming, together with hydrogen peroxide and the halide cofactor (Cl^-, I^-), one of the most potent microbicidal systems of PMNLs (De Chatelet et al., 1982; Thomas et al., 1988).

While monitoring chemiluminescent activity, we were mainly interested in the possibility of influencing the endogenic induction of free oxygen radicals, as well as the possibility of its application in radical chain reactions and oxidative processes in cell membranes and organelles. The "interface" between pro-oxidant and antioxidant processes is controversial and speculative. A significant role is played by the actual state of antioxidant activity as well as by interactions with other substances, when even antioxidants can, under certain circumstances, act in a pro-oxidant manner, which results in a significant change of their biological activity.

Extracts from various parts of the plants of genus *Echinacea* (*E. purpurea, E. angustifolia, E. pallida*) have become known primarily for their capability to strengthen the activity of an unspecified part of the immune system. North American Indians used these plants to treat febrile conditions and open wounds as well as insect or snake bites (Bauer, 1994). It has also been shown that an extract from the fresh plant, its top and root, acts as an immunostimulant when used in conditions such as the common cold, inflammatory processes, and malignant growths. The genus *Echinacea* contains substances similar in composition and character of effect. Pronounced immunostimulant, antibacterial, and virostatic effects have been associated primarily with polysaccharides, glycoproteins, alkamides, echinacoside (a glycoside with a pronounced analgesic effect), and caffeic acid derivatives (cichoric acid) (Bauer, 1996; Facino et al., 1995). The phagocytic activity PMNL in healthy volunteers was significantly enhanced by the alcoholic extract of *E. purpurea* radix (Melchart et al., 1995). The antiinflammatory effect is due to alkamides that inhibit the metabolism of the arachidonic acid (Müller-Jakic et al., 1994). The polysaccharide fraction increases the production of the "tumor necrosis factor" (TNF-α) and the induction of interleukins IL-1 and IL-6 (Roesler et al., 1991). On the German market, about 300 preparations containing *Echinacea* exist at present, indicated for use, for example, in atopic eczema, injuries, burns, and infections, as well as in polyarthritis and psoriasis. Most importantly, these preparations are recommended to strengthen the defensive capabilities, that is, immunity, of the organism (Bauer, 1994).

In our work, we tested *Echinacea* preparations using the chemiluminescence method to measure the activity of stimulated granulocytes. It is a dynamic test that demonstrates the formation of microbicidal substances in the phagocytes and evaluates their function. Specific surfaces on phagocytes form the first defense barrier against various pathological conditions of the macroorganism.

PROTOCOL

Test animals were female mice (6 weeks old), weighing 20 to 22 g, of the Balb/c strain (Biotest, Konárovice, Czech Republic). A commercial *Echinacea* product (distributed by Profitness, Ontario, Canada), consisting of the dried root and leaf of plants of several species, were dissolved into fine gelatinous matter in redistilled water, and applied by lavage. *Echinacea* was administered to the mice in daily doses of 83 mg/kg. Polymorphonuclear leukocytes were obtained from the peritoneum of six mice per group, 4 hours after giving 5 ml of glycogen by intraperitoneal injection. A veronal buffer at pH 7.3 containing two units of heparin per milliliter was used for washing out the peritoneum. The obtained cells were washed in the veronal buffer twice without heparin by centrifugation for 10 minutes at 300 g. The final concentration of cells was adjusted to 5.75×10^6 per ml in the veronal buffer without heparin. Each of four cm^2 polystyrene tubes contained a blended mixture of 0.4 ml of veronal buffer, 0.1 ml of dilute luminol, 0.4 ml of cell suspension, and 0.1 ml of 1% zymosan as stimulant (0.1 ml of veronal buffer replace zymosan in controls). Chemiluminescence activity was measured at room temperature at 5-minute intervals over a period of 90 minutes in an analytical luminometer.

RESULTS AND INTERPRETATION

In the first experiment (Figure 11.1), *Echinacea* was administered for 5 days and we began with the chemiluminescence investigation on the third day after termination. In the course of the following 4 days of testing we observed chemiluminescence values to be on the average 1.2 times greater in the group of mice treated with *Echinacea* compared with the control group. These values remained relatively consistent even on day 6 after termination of the *Echinacea* treatment. At each sampling interval, there was a statistically significant difference between the treatment and control groups of mice (third, fifth, sixth day $p < 0.01$; fourth day, $p < 0.05$).

In the second experiment (Figure 11.2), *Echinacea* was administered continuously for 16 days. The mice were tested daily for 5 days between Days 12 and 16. Chemiluminescence activity was an average 1.7 times higher (Day 16) than in the control group. After termination of *Echinacea* (Day 16) Days 19 and 22 after beginning treatment, the chemiluminescence values dropped to the levels similar to those found in Figure 11.1. All chemiluminescence values in the treatment group (Figure 11.2) were significantly higher ($p < 0.01$) than in the control group.

Chemiluminescence is an ideal test for monitoring the formation of free oxygen radicals in PMNL. On the one hand, the capability of PMNL to luminesce differs during inflammatory reactions and phagocytosis in response to bacteria, but on the other hand, it also reflects an increased risk, occurring with an overabundance of free radicals observed particularly in cases of insufficient antioxidant defense. Induction of oxygen radicals is relevant in relation to both the atherogenic and oncogenic processes. In the final stages of oncogenesis, the molecular switch is made that determines whether a cancer cell will continue its progression toward a tumor or, instead, destroy itself (apoptosis). The latter event involves cells of the immune system.

The administration of an extract from *E. purpurea* was followed by increased phagocytosis of *Candida albicans* by granulocytes and monocytes in healthy subjects as well as by an increase in

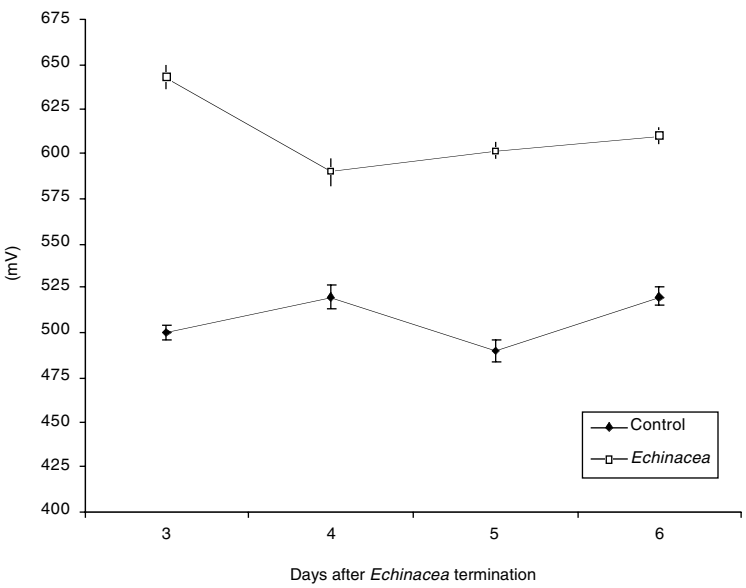

FIGURE 11.1 Chemiluminescence test (third to sixth days after termination).

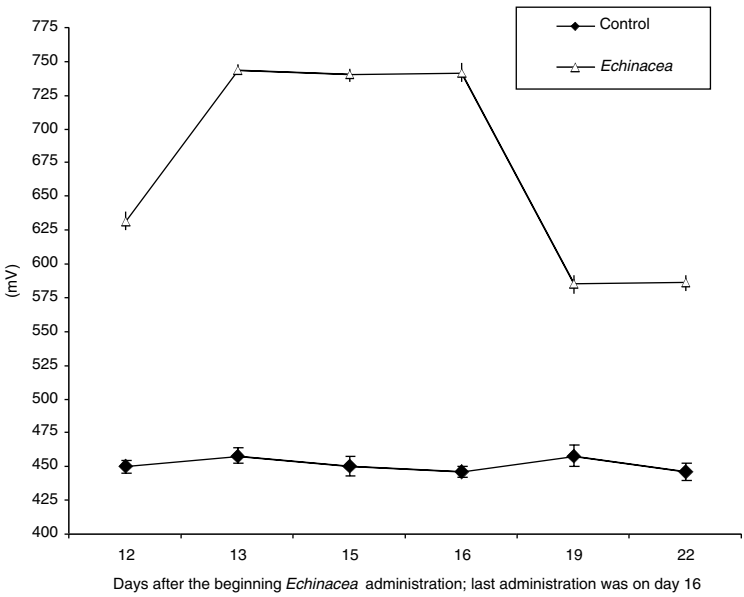

FIGURE 11.2 Chemiluminescence test (twelfth to twenty-second day of testing).

the chemotactic migration of granulocytes (Wildfeuer and Mayerhofer, 1994). In the macrophages that were influenced by *E. purpurea* and *E. pallida*, an increased production of TNF-α and the induction of the interleukins IL-1 and IL-6 and interferon were described by Steinmüller et al. (1993). Rininger et al. (2000) investigated the activation of macrophages using quantitative tests for the determination of TNF-α, IL-1, IL-6, and IL-10 derived from the macrophages. Similar results using *E. purpurea* were described by Burger et al. (1997) and Roesler et al. (1991), who observed that the administration of polysaccharides from *E. purpurea* also increased the spontaneous mobility of PMNL as well as their killing ability. The anticarcinogenic effect of *E. purpurea* is supported by the findings of stimulation of NK cells and their increased lytic function (Currier and Miller, 2001; See et al., 1997; Sun et al., 1999) as well as by the positive effect of root extract of *E. purpurea* when used in the *in vivo* treatment of leukemia (Currier and Miller, 2001). The alkamide fraction from *E. angustifolia* inhibits the activity of cyclooxygenase and 5-lipoxygenase, contributing in this way to the antiinflammatory effect. Facino et al. (1995) assumed that the protection of the organism against free radicals is due mainly to the polyphenols from the plants of the genus *Echinacea,* based on the ability of polyphenols to absorb reactive oxygen radicals. Extracts from roots and leaves of all three species of the genus *Echinacea* had antioxidant properties, absorbed free radicals (particularly the hydroxyl radicals), and reduced the peroxidation of lipids that results in the polyunsaturated fatty acids being transformed to alkanes, aldehydes, and other substances, some of which are toxic for the organism (Hu and Kitts, 2000; Sloley et al., 2001). The extracts from roots of plants of the genus *Echinacea* also suppressed the oxidation of human LDL (Hu and Kitts, 2000). Since oxidized LDL causes the progression of the atherogenic process, one can extrapolate that extracts from the genus *Echinacea* also have antiatherogenic effects. Rehman et al. (1999) studied the antigen-specific immunostimulant potential of *E. angustifolia* and recorded an increase in the immune reaction resulting in increased immunoglobulin production. A similar effect was also observed by Bodinet and Freudenstein (1999) using *E. purpurea* and *E. pallida* radix resulting in increased numbers of antibody-forming cells (PFC) as well as an increase in the titer of specific antibodies in tested animals. *Echinacea*, used traditionally in prophylaxis and treatment of respiratory infections, is a stimulant of nonspecific immunity, that is, the first line of

defense against cells affected by a virus or against cells transformed by a virus (Soon and Crawford, 2001; Sun et al., 1999). In our previous experiments (Šestáková and Turek, 1999), we found that dried roots and leaves from the plants of genus *Echinacea* can elicit increased activity in nonspecific immunity when administered in regular daily doses *in vivo*. After its discontinuation, the influence of the preparation declines, indicating that it is rapidly degraded *in vivo*. We regard the effect of *Echinacea* extracts as stimulating to PMNC when administered for a longer period (16 days), and the effect of a commercial *Echinacea* product can be modulatory even when the extracts are administered before the investigation.

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12 *In Vitro* Immunopharmacology of *Echinacea*

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INTRODUCTION

The medicinal herb *Echinacea* is a popular herbal remedy, reputed to be an immunostimulant. Three primary species of *Echinacea* are commonly employed in commercial preparations: *Echinacea angustifolia*, *Echinacea purpurea*, and *Echinacea pallida*. While there is a growing body of scientific evidence that supports the marketed uses of *Echinacea*, a tremendous deficiency still exists in our understanding of its pharmacological properties and human health benefits. This results from the various processing techniques employed for different species and sections of the plant that are harvested (roots and/or aerial parts) and their final formulation as a tincture, tablets/capsules, or teas. In fact, final product forms range from simple preparations of dried root and herb powders, pressed juice, or extracts standardized to a small percentage of constituent marker compounds. To further complicate matters, clinical trial results have demonstrated limited success, probably due to the lack of pharmacological characterization of the study material.

The application of *in vitro* experimental systems is fundamental to initial studies aimed at exploring the cellular responses associated with pharmacology and the potential efficacy of therapeutic agents. This is especially necessary for herbal medicines so that targeted clinical research can be conducted to further establish their credibility within the medical community. The goal of this chapter is to provide a concise yet comprehensive summary of the scientific evidence supporting the immunomodulating activities of *Echinacea* formulations and how *in vitro* bioassay methodologies have been applied to produce an *Echinacea* extract (CPT-121) with high immunostimulatory potency.

ECHINACEA CONSTITUENTS

There are four types of constituents purported as pharmacologically active molecules in *Echinacea* species: phenolic caffeic acid derivatives, glycoproteins, alkylamides/isobutylamides, and polysaccharides. In commercially prepared *Echinacea* extracts, the quantities of some of these constituents are measured to ensure that these presumed active ingredients are present. The development of "standardized" *Echinacea* extracts is a response to demands for more consistent end products and as a means to ensure consistency in desired effects. However, techniques that serve to enrich end

products for one class of constituents typically reduce or exclude others, with the exception of polysaccharides and glycoproteins, which are water soluble.

In Vitro Pharmacological Characterization of *Echinacea* Constituents

The most common constituents found in standardized extracts include polyunsaturated alkylamides or caffeic acid derivatives such as cichoric, chlorogenic, and caftartic acids. These compounds have been shown to inhibit cyclooxygenase and 5-lipoxygenase, key enzymes associated with inflammation via the production of prostaglandins and leukotrienes (Clifford et al., 2002; Müller-Jakic et al., 1994). Cyclooxygenase inhibition is the mechanism of action of nonsteroidal antiinflammatory drugs, such as indomethacin and acetaminophen, which are well known and tolerated to reduce fever and pain associated with colds and flu. The inhibition of cyclooxygenases could explain some of the benefits associated with *Echinacea*; however, the potency of individual *Echinacea* alkylamides is only fractional at concentrations of 100 μg/ml (Clifford et al., 2002). The phenolic caffeic acid derivatives may be more potent for this activity based on *in vitro* cellular assays measuring prostaglandin production from stimulated macrophage cells (Rininger et al., 2000). Phenolic standardized extract did inhibit prostaglandin production by approximately 40% at concentrations of 20 μg/ml (Rininger et al., 2000). In contrast, indomethacin, a commonly used pain reliever and fever reducer, yielded approximately 90% inhibition of prostaglandin production at concentrations 200-fold lower than the *Echinacea* concentrations tested.

Phenolic constituents and extracts have also been shown to possess potent free-radical scavenging activity, an antioxidant property that has been linked to improving immune function (Kim et al., 1997; Rininger et al., 2000). Table 12.1 shows the results of direct free-radical scavenging activity of various forms of *Echinacea* and extract constituents caffeic acid and chlorogenic acid. Interestingly, there is a wide range in potency among standardized preparations, which brings us to the question of standardization test methodology. *E. purpurea* herb preparations showed relatively little potency in this free-radical scavenging assay. In addition, cichoric acid has been described to

TABLE 12.1
Free-Radical Scavenging Activity of *Echinacea* Constituents, Phenolic Standardized Extracts, and *E. purpurea* Herb

Echinacea Material Tested	EC50 (μg/ml)
Caffeic acid	8.0
Chlorogenic acid	6.0
4% Phenolic standardized extract	20.0
4% Phenolic standardized extract	79.0
4% Phenolic standardized extract	139.0
4% Phenolic standardized extract	23.0
E. purpurea herb	144.0
E. purpurea herb	175.0

Note: Data shown represent the concentration needed to quench 50% of the free radical DPPH.

have HIV-integrase inhibitory properties, an activity that disables the virus's ability to replicate (Lin et al., 1999; Reinke et al., 2002; Robinson Jr. et al., 1996a, 1996b). Overall, these activities are not direct immunostimulatory activities. This is further supported by *in vitro* and *in vivo* studies assessing immune parameters in laboratory animals that have shown no immune-stimulating activity of chlorogenic and cichoric acid tested as single agents (Exon et al., 1998; Goel et al., 2002; Rininger et al., 2000).

In contrast to the limited *in vitro* experimental evidence of immunostimulatory activity of caffeic acid derivatives and alkylamides, there is consistent and convincing evidence for the role of *Echinacea* polysaccharides to directly stimulate immune cells. Wagner et al. (1988) and Steinmüller et al. (1993) have worked extensively to investigate the immunostimulatory effects of polysaccharides from *Echinacea* (Luettig et al., 1989; Roesler et al., 1991a, 1991b; Steinmüller et al., 1993; Stimpel et al., 1984; Wagner et al., 1988). These researchers were successful in isolating several polysaccharide structures, including a variety of arabinogalactans. The complex and high-molecular-weight (10 to 75 kDa) polysaccharides were found to directly activate nonspecific immune cell types such as monocytes, macrophages, and natural killer (NK) cells. *Echinacea* polysaccharide-induced stimulation of these cell types initiated cytokine production (TNF-α) and elevated phagocytic activity and oxidative burst, resulting in enhanced *in vitro* and *in vivo* killing of *Leishmania*, *Listeria*, and *Candida* pathogens (Luettig et al., 1989; Roesler et al., 1991a, 1991b; Steinmüller et al., 1993; Stimpel et al., 1984; Wagner et al., 1988). Importantly, the *in vitro* characterization of the polysaccharide activity was dose dependent and with potent stimulation occurring at concentrations ≤10 μg/ml. In addition, there is a likely mechanism of action for polysaccharide-induced stimulation of immune cell types through the binding and activation of cell surface receptors present on target immune cells. The *Echinacea* polysaccharides were subsequently shown to activate nonspecific immune cells when evaluated in animal models as well as human subjects (Roesler et al., 1991a, 1991b; Steinmüller et al., 1993). This characterization of *Echinacea* polysaccharides is the best demonstration of *in vitro* bioassay activity yielding reproducible *in vivo* pharmacological effects.

See et al. (1997) provided an independent laboratory confirmation of the immunostimulatory properties of aqueous whole herb extracts in *ex vivo* studies with human peripheral blood mononuclear cells from normal, chronic fatigue syndrome (CFS) and HIV-infected donors. This work showed that *Echinacea* enhanced endogenous NK function as well as antibody-dependent cellular cytotoxicity (ADCC) against human herpesvirus-6 infected cells from peripheral blood mononuclear cells (PBMCs) derived from each patient subset. *Echinacea*-induced responses were dose dependent and statistically significant at concentrations as low as 1 μg/ml. The overall stimulation observed was found to be greater in the immunocompromised cells derived from CFS and HIV-infected donors, at two- to three-fold for NK function and approximately five-fold for ADCC activity.

Rininger et al. (2000) was the third independent laboratory to corroborate the immunostimulatory activities of *Echinacea.* This research group employed a murine macrophage cell line and human PBMCs to conduct an immunopharmacological survey of *Echinacea* raw materials and finished products by comparing cytokine induction profiles as a measure of macrophage activation and human PBMC viability assays. The induction of TNF-α and nitric oxide proved to be the most sensitive macrophage biomarkers that were used to evaluate various commercial *Echinacea* raw materials and marketed products. The results demonstrated that the *Echinacea* herb and root powders possessed variable levels of stimulatory activity, and that standardized *Echinacea* extracts were devoid of this activity (Figure 12.1).

Subsequent evaluation of a dozen different lots of raw material, two of seven *E. purpurea* herb powders and one of five *E. purpurea* root powders had activity similar to the herb and root products first evaluated (Rininger et al., 2000). Testing of more than 40 individual herb and root powder raw materials found that approximately 30% of the raw material produced significant immunostim-

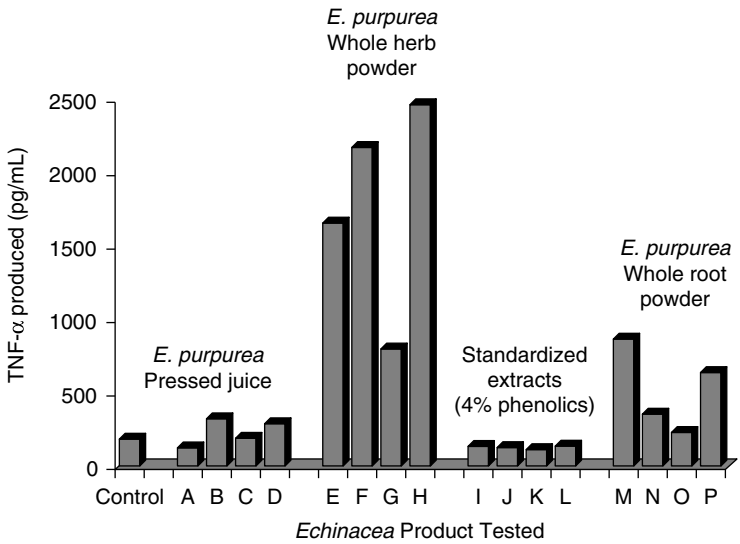

FIGURE 12.1 Macrophage activation following simulated digestion of various commercially available *Echinacea* products. Data represent the mean TNF-α secreted into cell culture supernatant after 24 hours of treatment with 20 µg/ml of *Echinacea*.

ulation detected through TNF-α and nitric oxide production (Rininger et al., unpublished observations, 2000). It is not surprising that the functional immunostimulatory activity is variable from lot to lot of material, which have multiple factors that can influence the presence of constituents, such as geographic location, seasonal growth conditions, harvest and processing procedures (milling and extraction), and storage conditions and time stored. The variability detected with *in vitro* product testing supports the use of bioassays to characterize products for quality control purposes.

Rininger et al. (2000) employed a simulated digestion methodology as a means to process *Echinacea* prior to testing after attempts using dimethylsulfoxide (DMSO) as an extraction solvent did not yield immunostimulatory activity. This sample preparation method was also attempted to emulate the conditions after oral consumption, the most common route of administration. In agreement with the findings from the aforementioned laboratories, the aqueous soluble material produced dose-dependent activation of the macrophage cells with significant activity in the low microgram per milliliter concentration range (Table 12.2).

The dose–response relationship for additional macrophage-secreting cytokines that included IL-1α, IL-1β, and IL-6 was also studied. It was found that these cytokines are also released; however, higher concentrations of *Echinacea* were needed (5 to 80 µg/ml) to induce them. These immunostimulatory attributes of *Echinacea* were far less potent and only transient compared to LPS, and may serve as an explanation of the low incidence of reported side effects from *Echinacea* administration.

Cytokines such as TNF-α, IL-1, and IL-6 were originally characterized as growth and activation factors for other immune cell types such as T and B lymphocytes, NK cells, and neutrophils (Billiau, 1986; DeChiara et al., 1986; Decker et al., 1987; Ghiara et al., 1987; Yokota et al., 1988). In order to demonstrate that *Echinacea* preparations could stimulate proliferation of various immune cell types, human PBMCs were treated with *Echinacea* without other stimulation, and cellular viability was assessed after 72 hours. In the absence of proliferative stimulation, PBMC viability dropped steadily over 72 hours (Reninger et al., unpublished observations, 2000). Different lots of *E. purpurea* herb that stimulated TNF-α production in the murine macrophage cell line significantly

TABLE 12.2
Dose–Response Relationship for Macrophage Activation by *E. purpurea* after Simulated Digestion Methodology

Echinacea Concentration (μg/ml)	TNF-α Produced (pg/ml)	Nitrites Produced (μM)
1280	12,713[a]	17.7[a]
320	8236[a]	16.0[a]
80	5856[a]	14.5[a]
20	2909[a]	11.3[a]
5	1255[a]	3.5[a]
1.25	620[a]	0.2[a]
0	312	0.0

[a] Statistically different from negative control at $p < 0.05$.

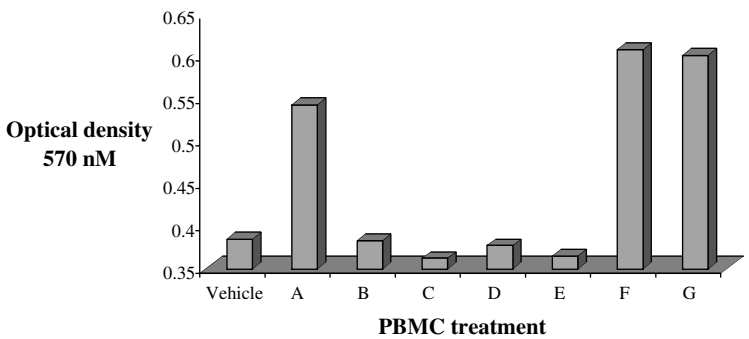

FIGURE 12.2 Enhancement of human PBMC cultures by *Echinacea*. The data represent the mean optical density readings from eight observations from a single human PBMC donor. *Echinacea* materials A, F, and G stimulated murine macrophages to produce TNF-α and significantly enhanced PBMC viability ($p < 0.001$) versus vehicle control. *Echinacea* preparations B through E did not stimulate TNF-α production from murine macrophages.

enhanced the viability of PBMCs. In contrast, *Echinacea* preparations that did not stimulate macrophage TNF-α production did not enhance PBMC viability (Figure 12.2).

Recently, the *in vitro* bioassays measuring TNF-α and nitric oxide production have been used to develop and characterize a new *Echinacea* extract preparation, CPT-121 (Columbia Phytotechnology, Pullman, WA). By employing the bioassays, the extract has been optimized to macrophage activation as a specific pharmacological mode of action. While the actual active constituents of this extract have not yet been elucidated, the biological activity measure can be employed for quality control purposes. In addition, results from the caco-2 cell monolayer–absorption model predict that the active components of this extract should be absorbed when administered orally.

The first step in the development of CPT-121 was to perform an assessment of macrophage immunostimulatory activity of different *Echinacea* extracts and fractionations. This included various *Echinacea* standard extracts, a raw herb powder, and preliminary fractionation of *E. purpurea* aerial parts as starting material for the development of CPT-121. As described previously in this chapter,

the results showed that *Echinacea* extracts standardized to phenolic or isobutylamide constituents and fresh pressed juice tested negative for macrophage activation (Figure 12.3).

From these preliminary results, conditions were modified to further optimize the extraction, concentration, and drying processes to produce extracts containing the immunostimulatory constituents. The resulting extracts from this series of experiments were tested for macrophage stimulation measured by TNF-α secretion (Table 12.3). Upon refining the process from this data, it was found that excessive heat diminished the immunostimulatory potential of the preparation and was consistent with prior experiments that evaluated extensive milling conditions of *Echinacea* herb powders (Reninger et al., unpublished observations, 2000). HPLC analyses for caffeic acid derivatives determined that these constituents were not present at significant levels in the CPT-121 extract

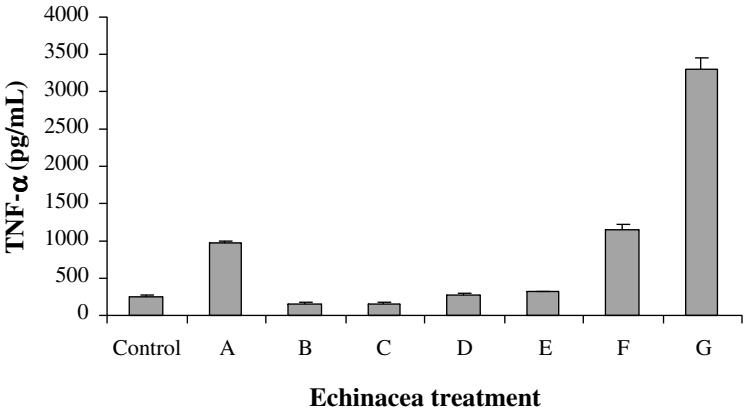

FIGURE 12.3 Macrophage stimulation following simulated digestion protocol of different preparations of *E. purpurea* herb extracts measured by TNF-α secretion. Data represent the mean TNF-α levels (pg/ml) ± standard deviation following 24 hours of stimulation. (A) Dried herb powder used to produce samples B, D, E, F, and G. (B) *E. purpurea* extract standardized to 4% phenolic compounds. (C) Isobutylamide extract from dried *E. angustifolia* roots. (D) HPUS ethanol extract. (E) *E. purpurea* fresh plant juicing (screw press). (F) Homogenate of fresh *E. purpurea* aerial parts. (G) CPT-121 pilot preparation.

TABLE 12.3
Extraction Optimization Experiments of CPT-121 Extraction Procedure for Immune-Stimulating Activity of *E. purpurea* Whole Herb Powder

Sample	Temperature Level	Residence Time	TNF-α (pg/ml)
1	Low	Low	7424
2	Low	High	7091
3	High	Low	5542
4	High	High	5649
E. purpurea Herb Starting Material	N/A	N/A	979
Control	N/A	N/A	192

Note: Data represent the mean TNF-α produced (pg/ml) of the cell culture supernatant after 24 hours of respective *Echinacea* extract stimulation. Each sample was used to treat three replicate wells of macrophage cells and the pooled supernatant run in three replicate ELISA wells.

N/A = not applicable.

(Figure 12.4). The CPT-121 extract contained over 80- and 10-fold less cichoric acid than the phenolic standardized extract and raw plant material, respectively. The production process for CPT-121 also virtually eliminated caftartic acid and reduced chlorogenic acid to undetectable levels (Figure 12.4). In addition, precautions were taken during processing to eliminate the possibility of bacterial growth to ensure that the subsequent immunostimulatory activity was not due to bacterial contamination. This was confirmed from subsequent testing of aerobic and anaerobic bacterial growth assays (Reninger et al., unpublished observations, 2000).

Testing of subsequent production batches of CPT-121 extract consistently demonstrated a similar level of the immunostimulatory potency. Dose–response experiments showed that the stimulatory activity was enhanced approximately 10-fold in comparison to the initial starting herb material (Figure 12.5).

Furthermore, this material was still approximately 20,000-fold less potent than bacterial LPS (Figure 12.5). In human PBMC viability assays, the activity of CPT-121 was found to be optimal at 100 ng/ml, 10-fold lower than the starting *E. purpurea* herb material. These data verified that the CPT-121 extract also stimulates human immune cells (Table 12.4).

Finally, to provide evidence that the active immunostimulatory constituents of the CPT-121 extracts would be absorbed from oral dosing, differentiated Caco-2 monolayers were employed as a predictive *in vitro* model of human absorption to determine the apparent permeability (P_{app}) of the active constituents (Artursson and Karlsson, 1991; Delie and Rubas, 1997; Yee, 1997). The use of the Caco-2 monolayer methodology has become prevalent in the pharmaceutical industry where it is used in combination with analytical detection to select potential lead compounds with good absorption and bioavailability (Taylor et al., 1997). This cell system has also recently been employed to determine the absorption of components of other medicinal herbal extracts (Kamuhabwa et al., 1999; Walgren et al., 1998). Nitric oxide production was quantified as a measure of macrophage activation produced from the extract constituents that permeated the monolayer. A dose–response treatment of RAW264.7 macrophage cells with CPT-121 was performed to estimate the absorption of the CPT-121 extract through differentiated Caco-2 monolayers via nitric oxide production. Utilizing the dose–response relationship, it was determined that approximately 0.9 µg of the CPT-121 extract activity had diffused through the Caco-2 monolayer. A P_{app} value was then calculated for the CPT-121 extract to be 12.8×10^{-6} (Table 12.5). Based on extensive analyses comparing P_{app} values derived from Caco-2 cells with drugs with known human absorption, this result would predict that the immunostimulatory constituents of the CPT-121 extract would be well absorbed (≥ 70%), based on the criteria set forth by Yee (1997).

SUMMARY

In this chapter, we provided a review of the scientific evidence from *in vitro* model systems to support the pharmacological activities of *Echinacea*. The majority of evidence to date indicates that *Echinacea* contains subsets of constituents that have antiinflammatory and antioxidant activities and those that are directly immunostimulatory to nonspecific immune cells. In addition, it has highlighted how *in vitro* bioassays have been applied to develop an *Echinacea* extract (CPT-121) optimized to immunostimulatory activity.

The design and implementation of *in vitro* experimental approaches are of central importance to define pharmacological profiles of herbal medicines and provide credible evidence for their efficacy to be assessed in clinical studies. Extracts from other popular herbal medicines including *Hypericum perforatum* (St. John's wort) and *Serenoa repens* (saw palmetto) that possess a defined *in vitro* pharmacology have subsequently been proven to have efficacy in human clinical trials as an antidepressant and for a treatment of benign prostate hyperplasia, respectively (Chatterjee et al., 1998a; Chatterjee et al., 1998b; Iehle et al., 1995; Müller et al., 1998; Plosker and Brogden, 1996;

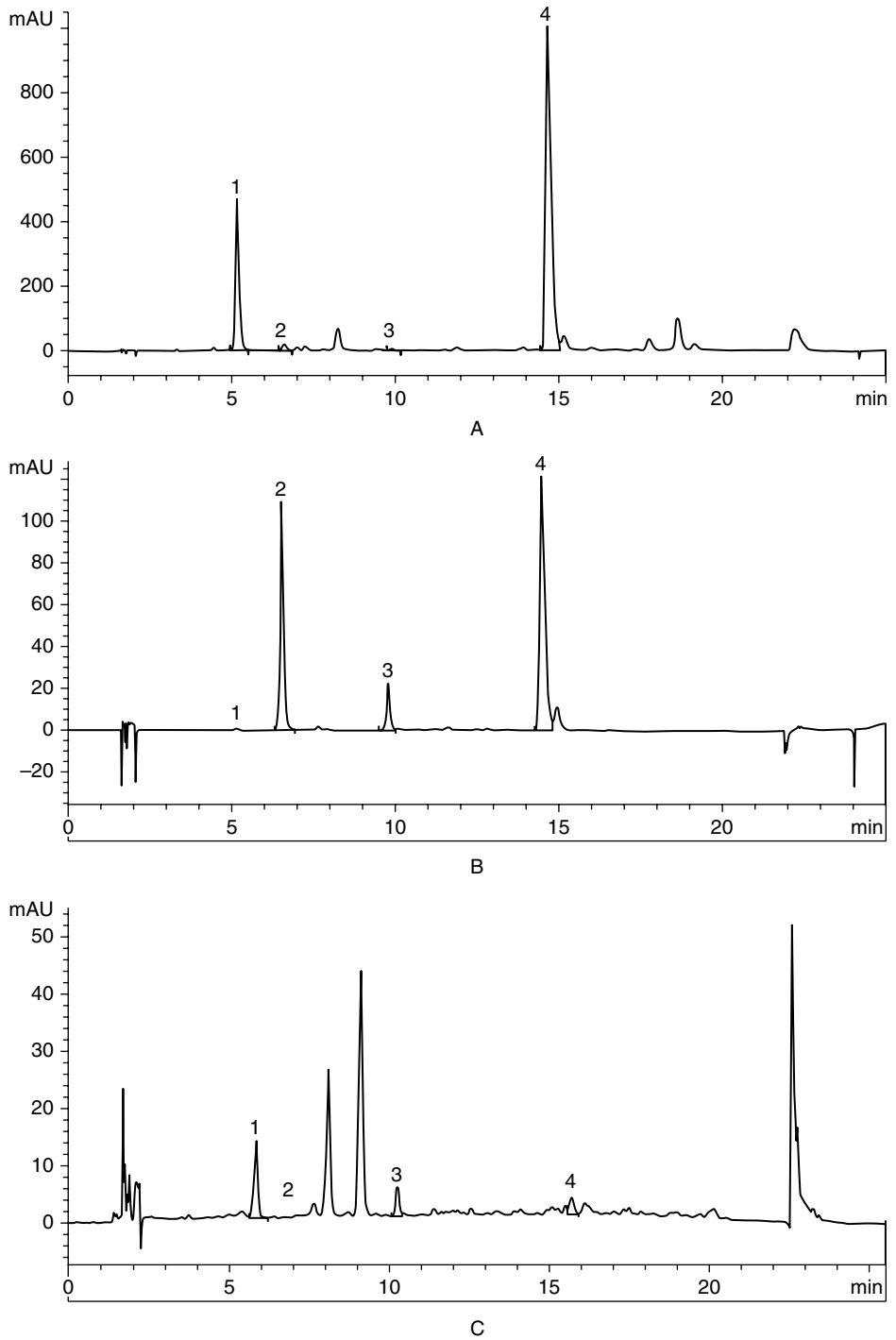

FIGURE 12.4 HPLC chromatogram profiles showing relative levels of *Echinacea* constituents from (A) phenolic standardized *Echinacea* extract; (B) raw *E. purpurea* herb; and (C) CPT-121 extract powder prepared from (B). Peak (1), caftartic acid; peak (2), chlorogenic acid; peak (3), echinocaside; peak (4), cichoric acid. Unnumbered peaks represent uncharacterized constituents. Analysis performed by Alpha Laboratories, Petaluma, CA.

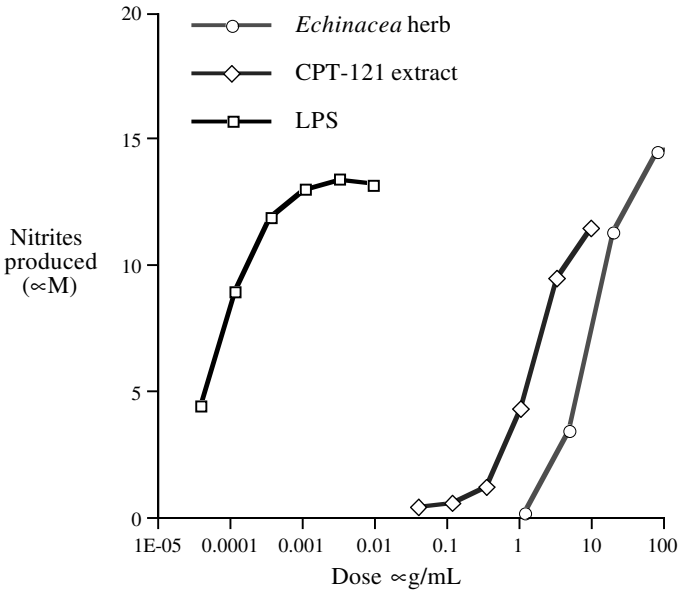

FIGURE 12.5 Dose–response comparison of macrophage activation profiles of the *Echinacea* herb starting material (open circles), the CPT-121 extract (open diamonds), and bacterial LPS (open squares). The *E. purpurea* herb starting material and CPT-121 extract were evaluated following a simulated digestion procedure. Data represent the mean for nitrites quantified from the cell culture supernatant after 24 hours of stimulation (N = 4, standard deviation < 3%).

TABLE 12.4
Enhancement of Human PBMC Viable Cell Numbers by *E. purpurea* and CPT-121 Extract Preparations Following Simulated Digestion Procedure

Cell Treatment	Mean O.D. 570 nM (± SD)
Control	0.457 ± 0.024
E. purpurea herb (1 µg/ml)	0.526 ± 0.014[a]
CPT-121 Lot 1 (0.1 µg/ml)	0.544 ± 0.025[b]
CPT-121 Lot 2 (0.1 µg/ml)	0.546 ± 0.019[b]
CPT-121 Lot 3 (0.1 µg/ml)	0.534 ± 0.026[b]
CPT-121 Lot 4 (0.1 µg/ml)	0.523 ± 0.018[b]

Note: Data represent the mean ± standard deviation of eight replicate-well optical-density readings resulting from an MTT-based cell proliferation assay.

[a] Statistically significant ($p < 0.001$) in comparison to placebo control cultures.

[b] Statistically significant ($p < 0.001$) in comparison to vehicle control cultures and not statistically different ($p < 0.01$) from parent *E. purpurea* herb sample.

O.D. = optical density; MTT = 3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

TABLE 12.5
Determination of CPT-121 Extract Permeability in the Caco-2 Monolayer Absorption Model Based on RAW264.7 Macrophage Nitric Oxide (Nitrites) Production

Cell Treatment	Nitrites Produced (μM)	Calculated P_{app} Value ($\times 10^{-6}$)
CPT-121 Extract (10 μg/ml)	27.7	
CPT-121 Extract (3.3 μg/ml)	20.5	
CPT-121 Extract (1.1μg/ml)	10.9	
CPT-121 Extract (0.37 μg/ml)	7.0	
CPT-121 Extract (0.12 μg/ml)	4.9	
No stimulation	4.7	
Caco-2 placebo basal	4.8	
Caco-2 CPT-121 extract basal	10.1	12.8
Permeability controls	Amount transported (μg)	
Phenol red (500 μg/ml)	< 1	< 1.0
Testosterone (50 μg/ml)	2.98	55.2

Notes: CPT-121 and placebo capsules for control were prepared through a simulated digestion protocol, and then diluted to 100 μg/ml in Hanks Balanced Salt Solution with glucose (HBSSg) and incubated in the apical compartment of Caco-2 monolayers for 1 hour at 37°C in a humidified, 5% CO_2 tissue culture incubator. Monolayer integrity was monitored by phenol red permeability ($P_{app} < 1 \times 10^{-6}$). The basal chamber fluid was lyophilized to concentrate the permeable material and reconstituted into RAW264.7 cell culture media. RAW cells plated in microtiter plates were then treated with reconstituted basal CPT-121 extract or control samples. Data represent mean nitrites produced (N = 5, standard deviation < 3%) from the respective treatments.

Raynaud et al., 2002). Unlike these two examples, other *Echinacea* extracts currently do not have a truly defined pharmacology. Therefore, it is not surprising that the clinical effectiveness of *Echinacea* preparations for the treatment (to provide faster resolution), prevention, or alleviation of symptoms of colds and flu is inconclusive (Barrett et al., 1999; Melchart et al., 1994; Melchart et al., 1998; Stuart, 1979). Continued research and definition of *Echinacea* preparation pharmacology will provide for better biomarkers of efficacy to be applied in clinical research to define the benefits and appropriate use of this popular herbal medicine.

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13 Bioassays of *Echinacea* Extracts and Commercial Products

Pamela S. Coker and N. Dwight Camper

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INTRODUCTION

Echinacea species (coneflowers) are important plants in both the pharmaceutical and ornamental industries. *Echinacea*, a genus of the Aster family, is represented by nine species found only in the U.S. and south-central Canada. More than 200 pharmaceuticals are made from coneflowers in Germany alone (Foster and Duke, 1990). Commercial West German *Echinacea* preparations utilize extracts of aboveground parts and roots. Purple coneflower (*Echinacea purpurea* L.) is widely cultivated in gardens and grows wild in some places (Hobbs, 1989).

Echinacea was introduced in the U.S. market in 1871 by a patent medicine vendor in Nebraska (Tyler, 1993). Traditional medicinal uses of this species include an immunostimulant for flu and colds, wound healing, and throat infections. Most frequent major therapeutic and prophylactic applications are for chronic and recurrent infections of respiratory and urogenital organs, chronic inflammations/allergies, tonsillitis and sinusitis, infected wounds, eczema and psoriasis, chronic bronchitis and prostatitis, and malignant diseases (Bauer and Wagner, 1991). Both cortisone-like

and immunostimulant activity have been confirmed (Bauer and Wagner, 1991; Hobbs, 1989). Formulations of *Echinacea* are found in salves, tinctures, capsules, or teas (Foster and Duke, 1990). Active ingredients include cichoric acid, echinaceine, echinolone, and echinacoside.

Several members of *Echinacea* are endangered species; thus, collection of plants for research and extraction of pharmaceuticals is not allowed, or at best restricted (Murdock, Southeast Region, U.S. Fish and Wildlife Service, Georgia, 1994, personal communication). Successful *in vitro* culture protocols have been established for *E. purpurea* (Coker, 1999; Coker and Camper, 2000).

Assessment of plant extracts or commercial products to verify folklore, anecdotal, or other types of information (ethnobotanical, observations, or serendipity) requires some type of initial screening followed by clinical studies. Detection of biologically active components in a medicinal plant extract by carefully designed screens or bioassays is an effective strategic plan to verify reported or claimed activity or traditional use. Screening bioassays must meet several criteria; they must be rapid, convenient, reliable, inexpensive, sensitive, require little material, and be able to identify a broad spectrum of activities. These criteria were recently verified for an antitumor bioassay, the potato tumor induction assay, and the assay would detect chemicals that disrupted the cell cycle at any point (Coker et al., 2003). Bioassays can also be used to direct extract fractionation that may lead to identification of active ingredients in a crude extract that exhibits specific biological activity. Additionally, bioassay results can identify extracts, or fractions thereof, that should be included in clinical studies.

Bioassay tests can provide valuable information about a plant extract or fraction and its biological activity. While bioassays do not deal specifically with the interactions between the organism and the extract or drug, a modification within the bioassay can assess the biotransformation and its subsequent effect on biological activity. This will provide some information about whether the organism will transform the extract or drug rendering it inactive biologically or converting it to a more active chemical form. In studies described herein, an additional treatment used a human microsomal fraction, which was rich in cytochrome P450 enzyme activity. This enzyme activity is found most abundantly in the liver, but is also found in small amounts in other body tissues. Cytochrome P450 enzymes are involved in biotransformation of drugs and other compounds in the body (detoxification and metabolism). Cytochrome P450 oxidative reactions result in a more water-soluble chemical, thus facilitating elimination from the body (Cupp and Tracey, 1998). Another potential result is transforming a chemical from a toxic form to a nontoxic form, or to convert a chemical from a tumor inducer to a tumor inhibitor. Thus, inclusion of a microsomal fraction treatment in the bioassays reported herein was intended to simulate passage of the plant extract through the body. Bioassays used in these studies were classified as "bench-top" bioassays that did not involve live animals or human subjects.

Selected bench-top bioassays used in studies with purple coneflower are discussed below. The discussion focuses on the type of information that can be gained, and how it might lead to further bioassay-directed fraction and clinical studies. Results obtained with various purple coneflower extracts and commercially available products are summarized.

ANTIMICROBIAL ACTIVITY

Extracts from purple coneflower are reported to have antimicrobial properties, as well as antiviral and immune-stimulating properties. Antimicrobial activity has been attributed to two chemical families, the polysaccharides and alkyl amides. Extracts may be used topically, orally, intravenously, or intramuscularly, and have been tested in Europe against upper respiratory tract diseases, wounds, urinary tract infections, *Herpes simplex* virus, and influenza. However, these tests were not all performed with sufficient quality control to merit acceptance in the U.S. (Hobbs, 1990).

The Kirby–Bauer sensitivity test (diagrammatically illustrated in Figure 13.1) was used to test various purple coneflower fractions and extracts with several different bacteria. Filter paper discs

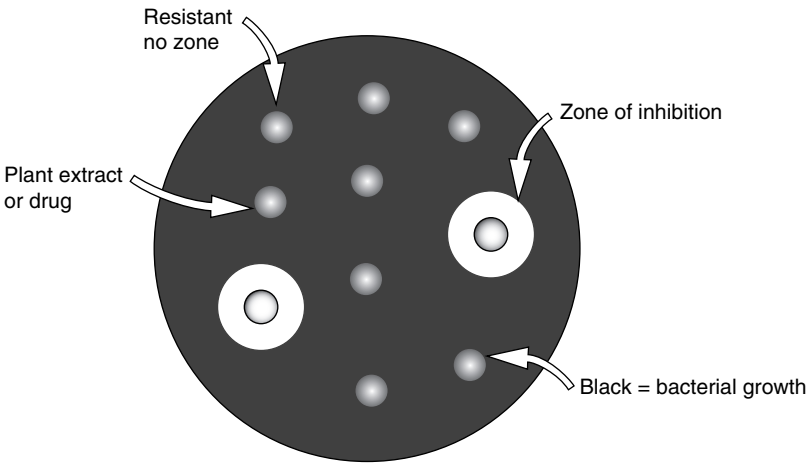


FIGURE 13.1 Schematic drawing of a Kirby–Bauer sensitivity test. Filter paper discs shown in grey; zones of inhibition are in white.

are saturated with the test material and placed on an agar medium inoculated with the bacterial suspension. Clear zones around the discs indicate that as the test material diffuses from the disc, bacterial growth is inhibited (zone of inhibition). The presence of no clear zones around the discs indicates no inhibitory response. Measurement of the inhibition zone provides quantitative data enabling evaluation of test sample efficacy; for example, the larger the zone of inhibition, the more inhibitory or active the test sample.

Experimental Protocol

Samples tested included the following:

Three organic solvent fractions (n-butanol, methanol, and hexane) of bulk plant material (consisting of roots, stems, leaves, and flower tops purchased locally)
A crude tea prepared from steeping bulk plant material in water for 15 minutes
Four commercial products:

1. A tincture containing *E. angustifolia* and *E. purpurea* root (designated EC-1)
2. An alcohol-free sample in glycerine containing *E. angustifolia* root (designated EC-2)
3. A tincture containing *E. angustifolia* root, *E. purpurea* root, flower head, and seed, *Hydrastis canadensis* root, *Berberis aquifolium* root, *Berberi*s spp. bark, *Hypericum perforatum* buds, and propolis extract (designated EC-3)
4. A tincture containing *E. angustifolia* root and *E. purpurea* root, flower head, and seed (designated EC-4)

Bacteria tested included *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Filter paper discs were saturated with each test sample and placed on plates inoculated with one of the test bacteria. For quality control, antimicrobial susceptibility test discs (QC discs) were obtained from Becton Dickinson. These disks were impregnated with drugs currently in use at specific concentrations, and were accompanied with expected susceptibility results for each organism tested (Figure 13.2).

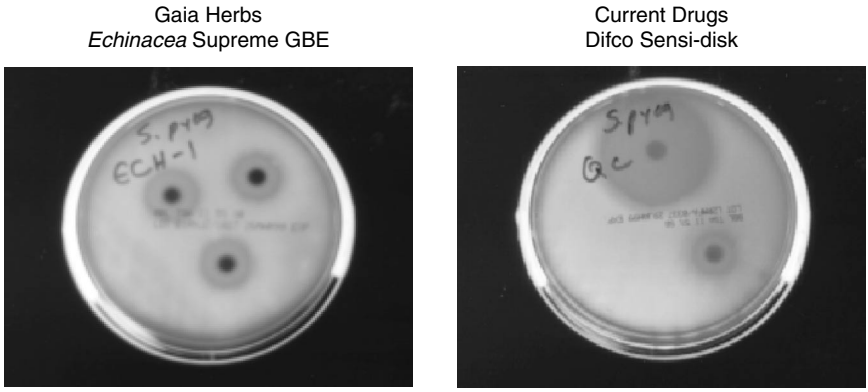


FIGURE 13.2 Kirby–Bauer sensitivity. Petri dish inoculated with *S. pyogenes* shows zones of inhibition by commercially extracted *E. purpurea* products (left). Petri dish inoculated with *S. pyogenes* shows zones of inhibition around Difco Sensi-disk control drugs (right).

Results and Discussion

Bacterial suspensions were standardized (absorbance versus colony-forming units, an indication of bacterial growth) in order to establish an inoculum, which is critical for quality control of diffusion disc analysis. No effect of the commercial products on *E. coli* was observed; however, three of the commercial products were active against *S. pyogenes* (Table 13.1). For EC-1 (tincture of *E. angustifolia* and *E. purpurea* root tissue) and EC-4 (tincture of *E. angustifolia* and *E. purpurea* root, flower head, and seed), the inhibition zones (activity) averaged 14.4 mm and 13.4 mm, respectively, which is comparable to the activity of the prescription drug sulfizoxazole (Table 13.1). The inhi-

TABLE 13.1
Zone of Inhibition for Each Drug or Sample Tested in Disk Diffusion Assay

Drug/Sample	Bacterium Tested	
	E. coli	***S. pyogenes***
Tetracycline-30	27[a]	22
Carbenicillin-100	27	42
Cephalothin-30	18	32
Gantrisin-25 (sulfasoxizole)	25	13
Ampicillin-10	16	32
Chloramphenicol-30	28	24
EC-1	0	14
EC-2	0	0
EC-3	0	6
EC-4	0	13

Notes: *Echinacea* samples tested included a tincture mixture containing *E. angustifolia* and *E. purpurea* root (EC-1); glycerine extract containing *E. angustifolia* root (EC-2); tincture containing *E. angustifolia* root, *E. purpurea* root, flower head, and seed, *Hydrastis canadensis* root, *Berberis aquilolium* root, *Berberis* spp. bark, *Hypericum perforatum* buds, and propolis extract (EC-3); and tincture containing *E. angustifolia* root and *E. purpurea* root, flower head, and seed (EC-4).

[a] Zone of inhibition in mm.

bition zone for sample EC-3 (tincture mixture) was 6 mm, one half that of sulfizoxazole. QC disc results were consistent with the expected values (Table 13.1). A minimal amount of direct antimicrobial action was observed for EC-3 against *S. pyogenes*. EC-1 and EC-4 produced activity against *S. pyogenes* comparable to that of the prescription drug sulfizoxazole. No direct antimicrobial activity against *E. coli* or *S. pyogenes* was observed for any organic solvent fraction or the crude aqueous extract (tea) from bulk material tested.

ANTINEOPLASTIC ACTIVITY: POTATO TUMOR INDUCTION ASSAY

The potato tumor induction assay measures the ability of an extract or chemical to inhibit tumor formation. This assay uses *Agrobacterium tumefaciens* as a tumor initiator; the bacterium is a gram-negative rod and is the causative agent of crown gall disease in plants (Agrios, 1997; Anand and Heberlein, 1977; Lippincott and Lippincott, 1975). Crown gall disease causes a mass of tissue (callus) bulging from stems and roots of woody and herbaceous plants. These masses (tumors) may be spongy or hard, and may or may not cause a deleterious effect on the plant.

During infection of plant material with *A. tumefaciens*, a tumor-producing plasmid (Ti-plasmid) found in the bacterial DNA is incorporated into the plant's chromosomal DNA. When plant tissue is wounded, it releases phenols and other chemicals that stimulate the Ti-plasmid. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in the formation of tumors that are similar in nucleic acid content and histology to human and animal cancers (Agrios, 1997). Tumorigenesis in plants and animals involves similar mechanisms and common nucleic acid components (Agrios, 1997; Ferrigini et al., 1982). The tumor tissue in plants is known as callus tissue and may eventually differentiate into vascular tissue, just as animal tumors will mutate to produce blood vessels.

The potato tumor induction assay may identify agents that damage or stop the synthesis of DNA, preventing cellular division. It may identify compounds that stop mitosis, also preventing cellular division, thereby halting tumor growth. For example, etoposide, a semisynthetic derived from podophyllin, directly damages the DNA in the cell nucleus; vincristine and vinblastine are active in blocking the synthesis of the spindle in mitosis, where paclitaxel is active in blocking disassembly of the mitotic spindle (Riley, 1999).

Ferrigni et al. (1982) used the potato tumor induction assay to determine possible antitumor activity of several plant extracts (e.g., members of the *Euphorbiaceae*) with *A. tumefaciens* as the tumor initiator. In 1993, McLaughlin et al. used the potato tumor disc assay to evaluate several other plant extracts. In both of these studies, the potato tumor induction assay was compared to the 3PS *in vivo* tumor assay, the standard test for new antitumor agents. In the 3PS *in vivo* tumor assay, leukemic mice are treated with possible antitumor agents (McLaughlin et al., 1991, 1993). Life span differences of the leukemic mice compared to healthy mice are used as a measure of antitumor activity. A major problem in using the 3PS *in vivo* tumor assay is that high concentrations of antitumor agent often prove fatal to the subjects. The potato tumor induction assay eliminates this problem (Ferrigni et al., 1982; McLaughlin et al., 1991, 1993).

This assay is sensitive to the promotion and progression stages of carcinogenesis. Stage 1 (initiation stage) involves a mutation in a single cell that leads to increased proliferation. Stage 2 (promotion) involves reversible growth stimulation and requires promoting factors that are not carcinogenic themselves, but cause abnormal cell proliferation. This stage may be reversed if promoting factors are removed. Stage 3 (progression) involves irreversible growth; cells become immortal and proliferate at an exaggerated rate. Stage 4 involves invasion and metastasis, where cells invade underlying tissue, break off, and move to other areas.

Experimental Protocol

Plant samples consisted of an ethanolic tincture and a glycerol extract that were derived from whole *E. purpurea* plants, and a capsule derived from roots of *E. purpurea* and *E. angustifolia*. These products were purchased from a local health food store.

Discs were cut from disinfested Russet potato cylinders and placed in 24-well culture plates containing water agar. Standardized suspensions of *A. tumefaciens* were added to the wells; controls included the bacterium alone, camptothecin (a known tumor inhibitor), and solvents with and without the bacterium. After 12 days of incubation at room temperature, the discs were stained with Lugol's reagent (I_2KI), which reacts with starch in the disc. The tumors do not react with Lugol's reagent and appear as white- to cream-colored masses against a dark purple or black background, and can be counted using a dissecting microscope.

Results and Discussion

The potato tumor induction assay was used to determine whether *Echinacea* products inhibit or promote tumor formation. *A. tumefaciens* alone served as a negative inhibitory control and with camptothecin at 0.1 ppm served as a positive inhibitory control (Figure 13.3). The ethanolic tincture (E1) at 0.1 ppm, 1.0 ppm, and 10 ppm showed no activity compared with *Agrobacterium* alone (negative inhibitory control), but was significantly different from the camptothecin sample (positive inhibitory control) (Table 13.2). Glycerol extracts (E2) at 0.1 ppm, 1.0 ppm, and 10 ppm were not significantly different from each other, but E2 at 0.1 ppm (13 tumors observed) was significantly different from all concentrations of E1 (18.0 to 18.6 tumors observed) and the negative inhibitory control (11.9 tumors observed). E2 at 1.0 ppm significantly inhibited tumor induction over the negative control, but inhibitory activity was not significant at 10 ppm. The dried root complex (E3) dilutions (0.1 ppm, 1.0 ppm, and 10 ppm) were not significantly different from each other or from the positive inhibitory control, but were significantly different from the negative inhibitory control (11.9 tumors observed on average). As a group, E3 exhibited an average of 14.0 to 15.6 tumors observed, whereas *Agrobacterium* alone (negative inhibitory control) induced an average 20.4 tumors. All dried root

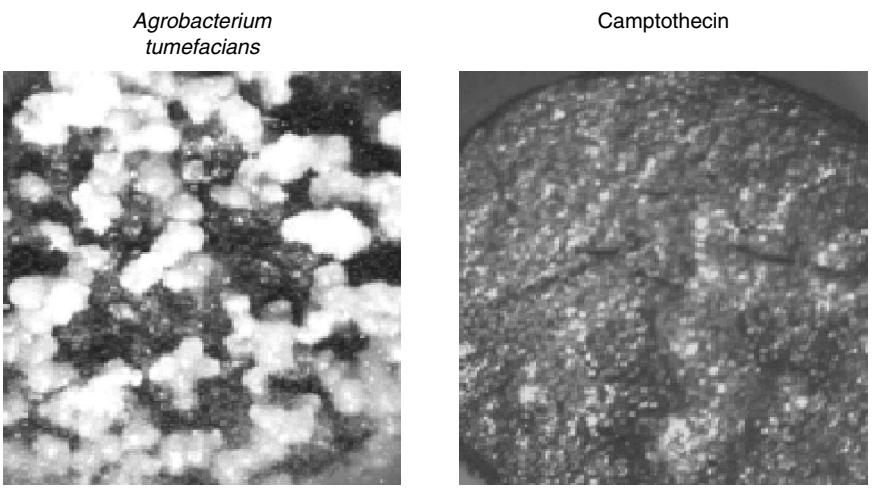


FIGURE 13.3 Tumors induced by *Agrobacterium tumefaciens* (transformation control) (left). Tumors appear as white to cream-colored nodules on the surface of the potato disc (dark purple or black in the photo above). No tumors appear when camptothecin was added (positive inhibitory control) (right).

TABLE 13.2
Number of Tumors Observed in Potato Tumor Induction Assay When Treated with Three *E. purpurea* Extracts, *Agrobacterium* Alone, and Camptothecin

Sample*	Concentration (ppm)	Average Number of Tumors**
E1	0.1	18.0 abc
E1	1.0	18.5 ab
E1	10	18.6 ab
E2	0.1	13.0 de
E2	1.0	15.8 bcde
E2	10	16.6 abcd
E3	1.0	14.3 bcde
E3	0.1	14.0 cde
E3	10	15.6 bcde
Agrobacterium	Not applicable	20.4 a
Camptothecin	0.1	11.9 e

* E1 = ethanolic extract; E2 = glycerol extract; E3 = dried root complex.

** Significant difference as determined by a *t*-test (least-squares difference) using the SAS computer program. Values followed by one or more of the same letters are not significantly different.

complex samples (E3) were inhibitory to tumor production in this assay and were similar to the camptothecin control. The ethanolic tincture (E1) was not inhibitory; whereas the glycerol extracts at 0.1 ppm and 1.0 ppm were significantly inhibitory, but not active at 10 ppm. Bauer et al. (1990, 1991) showed that root extracts contained the most active constituents in the plant.

The activity in the glycerol extract and dried root complex sample was comparable to that of the camptothecin control, supporting further investigations into their use as antineoplastic agents. Although no inhibitory activity for ethanolic extracts was observed in this assay, the positive results for the other *Echinacea* products encourage further investigation of the product. This variability in results may be due to differences in extraction procedures, shelf life, or plant variability between each form tested. The variability between forms also confirms that standardization is the key to reliable herbal products. Although this assay is able to detect antineoplastic activity, it can only measure activity on the cell cycle. It does not address antineoplastic activity pertaining to receptor sites or those agents that may stop the development of blood vessels.

ANTINEOPLASTIC ACTIVITY: CELL PROLIFERATION ASSAY

The MTS (dimethylthiazole) assay was used to measure the ability of *Echinacea* products to stimulate or arrest metabolic activity of tumor cells. The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay manufactured by Promega is a colorimetric method used to measure dehydrogenase activity in metabolically active cells. Specifically, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophen-yl)-2H-tetrazolium, inner salts (MTS) with the electron-coupling reagent, phenazine methosulfate (PMS), is reduced to formazan in the presence of metabolically active cells. Absorbance of reduced formazan is proportional to the number of viable cells in culture.

These assays have been used to test tumor-inhibiting properties of a number of plants including *Camellia sinensis* (green and black tea), *Allium sativum* (garlic), and *Solanum muricatum* (nightshade), among others. Human cancer cell lines used in the assay include those from breast, prostate, lung, liver, colon, bladder, and liver. Normal human cell lines used in the assay include those from skin fibroblast, lymphocyte, prostate, umbilical vein endothelial, and lung fibroblast cells. Tumor-

inhibiting properties studied include apoptosis; induction of phase II enzymes, glutathione-S-transferase, quinone reductase; and DNA breakages in G1 phase of the cell cycle, among others (Ikemoto et al., 2000; Lee et al., 1999; Lu et al., 1996; Steele et al., 2000; Swamy and Tan, 2000). One study used a dimthylthizole assay to investigate the activity of plant extracts against the intestinal parasite *Giardia lamblia* (Ponce-Macotela et al., 1994). The assay used in this study was Promega's CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay.

Experimental Protocol

Plant samples were from the same source as used in antineoplastic activity bioassays.

Cell Cultures

Cell lines used in this study were purchased from American Type Culture Collection (ATCC) and consisted of SiHa cervical cancer cells (ATCC #HTB-35) and MCF7 breast cancer cells (ATCC #HTB-22). Standardized cell suspensions (diluted to a specific cell number per unit volume) were treated with test materials (Coker, 1999).

MTS Assay

Ninety-six well plates were inoculated with specific concentration of cell suspension (1×10^5 cells/ml) incubated in a CO_2 (5%) incubator at 37°C for 48 to 72 hours. After incubation, MTS/PMS solution was added and the plate incubated for another 1 to 4 hours; absorbance was determined at 490 nm.

Each sample was also treated with an S9 human liver microsome fraction. The cells were cultured and the assay performed as previously stated with the addition of 50:l 4% human S9 mix to each tube before the cells were added.

Results and Discussion

The ethanolic tincture at 0.1 ppm, 1.0 ppm, and 10 ppm (E1A, B, C); the glycerol extracts at 0.1 ppm, 1.0 ppm, and 10 ppm (E2A, B, C); and the root complex sample at 0.1 ppm, 1.0 ppm, and 10 ppm (E3A, B, C) had no effect on the cervical cancer cells or the breast cancer cells tested (Table 13.3 and Table 4). Although E1 and E3 samples at 0.1 ppm and 1.0 ppm, respectively, with S9 activation significantly reduced the metabolic activity in the cervical cancer cells when compared to cells without S9, there was no significant difference between them and the cells with S9 (Table 13.4). Metabolic activity of the cervical cancer cells was higher when incubated with sample E1 treated with S9 at 1.0 ppm than the cells with S9 added (Table 13.4). Sample E1 with S9 at 0.1 ppm and sample E3 with S9 at 10 ppm induced a significant increase in metabolic activity of the breast cell line as compared to the cells with S9 added (Table 13.3). This increase in metabolic activity upon addition of S9 and the ethanolic tincture at 0.1 ppm and the dried root complex sample at 10 ppm is consistent with results obtained by Harrington-Brock et al. (1998). Although treatment of E2 and E3 samples at 0.1 ppm and E3 at 1.0 ppm appeared to significantly reduce metabolic activity of S.Ha cells (Table 3.4), activity was not reduced more than that of the controls (cells in media only).

In conclusion, the ethanolic tincture had no effect on the metabolic activity of SiHa or MCF7 tumor cells regardless of concentration unless S9 was added. In the presence of S9, the ethanolic tincture at 0.1 ppm inhibited metabolic activity of SiHa tumor cells, but not more so than cells treated with S9. However, SiHa tumor cells exposed to 1.0 ppm of the ethanolic tincture with S9 exhibited significantly higher activity than that of cells treated with S9. The ethanolic tincture with S9 appeared to significantly increase metabolic activity of MCF7 tumor cells at 0.1 ppm over cells treated with S9, but had no effect at any other concentration.

TABLE 13.3
Metabolic Activity of MCF7 Breast Cancer Cells When Exposed to *Echinacea* Products with and without S9 Treatment

Sample	Concentration (ppm)	Optical Density, MCF7 Cells*	Optical Density, MCF7 Cells + S9 Treatment*
Ethanolic Extract	0.1	0.93 abcd*	0.84 cdefg
	1.0	0.97 ab	0.80 fgh
	10	1.01 a	0.78 gh
Glycerol Extract	0.1	0.79 fgh	0.67 j
	1.0	0.90 bcde	0.77 ghi
	10	0.94 abc	0.83 egh
Dried Root Complex	0.1	0.89 bcdef	0.65 j
	1.0	0.82 efgh	0.68 ij
	10	0.96 ab	0.90 bcde

* Significant difference based on Duncan's multiple range test for optical density. Values within a column followed by one or more of same letters are not significantly different. Optical density of control cells without S9 was 0.83 and with S9 was 0.72.

TABLE 13.4
Metabolic Activity of SiHa Cervical Cancer Cells When Exposed to *Echinacea* Products with and without S9 Treatment

Sample	Concentration (ppm)	Optical Density, SiHa Cells	Optical Density, SiHa Cells + S9 Treatment
Ethanolic extract	0.1	1.44 cdef*	1.27 f
	1.0	1.77 ab	1.90 a
	10	1.62 bc	1.40 cdef
Glycerol extract	0.1	1.40 cdef	1.34def
	1.0	1.59 bc	1.45 cdef
	10	1.73 ab	1.58 bc
Dried root complex	0.1	1.55 bcd	1.39 cdef
	1.0	1.49 def	1.45 cdef
	10	1.54 bcde	0.90 ef

Note: Significant difference based on Duncan's multiple range test for optical density. Values within a column followed by one or more of the same letters are not significantly different. Optical density of control cells without S9 was 1.57 and with S9 was 1.49.

MUTAGENICITY ACTIVITY

A mutagen is an agent that causes genetic mutations, and carcinogenesis is the development of cancer. Cancer originates from genetic changes in a single cell.

The Muta-ChromoPlate™ assay is a modified Ames test used to measure the mutagenicity potential of a substance. Both the Ames test and the Muta-ChromoPlate assay are reverse-mutation assays. In both, tester strains of *Salmonella typhimurim*, which require histidine for growth, are exposed to various test samples. The ability of the bacterium to revert to non–histidine-dependent growth in the presence of a sample is a measure of the mutagenicity potential of that sample.

The Ames test involves direct counting of bacterial colonies, on solid agar. The Muta-ChromoPlate assay incorporates bromcresol purple as an indicator of pH, which is further correlated to the presence of acid-producing bacteria in liquid culture. The *Salmonella* strain used in this assay produces acid. Both tests measure the fluctuation between growth of the original colonies (tester strain) and colonies that grow after histidine exhaustion (reverse mutants). This phenomenon is based on work done by Luria and Delbruck (1943) in liquid cultures that was modified by Hubbard et al. (1984) and Ames et al. (1975).

Broth fluctuation tests similar to the Muta-ChromoPlate assay have been used by many researchers. They are thought to be more sensitive than the Ames test performed on solid agar plates because a smaller population of tester bacteria may be used (Hubbard, 1984). Angerer (2001) evaluated fluctuation experiments and concluded that they were as good or better than other methods for measuring mutation rates. Green et al. (1976) used broth fluctuation analysis to look at the mutagenic potential of mitomycin C, dichlorvos, and K_2CrO_4, and concluded that fluctuation analysis was more sensitive than conventional tests identifying mutagens at concentrations from 0.0015 m:g/ml to 5 m:g/ml. Fluctuation analysis was used by Cole et al. (1976) to measure induced mutations in mouse lymphoma cells, and was found to be a more sensitive indicator of mutagenesis.

Experimental Protocol

The Muta-ChromoPlate assay was used to measure the mutagenicity potential of *Echinacea* products. Plant samples are from the same source as used in the antineoplastic activity bioassays.

The tester strain of *S. typhimurim* contained a mutation of the hisG46 gene as well as a resistance factor, making it a histidine auxotroph with increased sensitivity to mutagenesis. The test was performed as directed according to Muta-ChromoPlate package directions (EBPI Inc, Ontario, CA). A positive control consisted of 2-amino-anthracene (2AA), a known mutagen. Other controls consisted of the bacterium plus S9, solvent controls, and the bacterium alone. After 5 days, the color of each well was recorded: yellow or partially yellow meant positive (reverse mutants produced); and purple meant negative (no mutants produced). The 2AA produced a yellow color (reverse mutants produced).

Results and Discussion

Ethanolic tincture (E1), glycerol extract (E2), and dried root complex capsules (E3) without activation with S9 were no more mutagenic than the background. All extracts, regardless of source, ethanolic, glycerol, or dried root, were mutagenic when treated with S9. The S9 alone increased the number of mutations over the water blank (Table 13.5). This may be due to the fact that S9 served as a histidine source, or there was a small amount of a mutagenic substance in the S9 (J. Rundell, Moltox Inc., personal communication, 2002). In the preparation of S9, human liver was harvested at autopsy from five individuals and the homogenate pooled. In the lot number used for this assay, one of the donors was positive for tobacco, alcohol, and cannabis, which supports the idea that there may have been a mutagen present in the pooled S9. Although the S9 did increase the number of background mutations, the increase observed with samples was significantly greater. All three extracts with S9 added (E1S9, E2S9, E3S9) were significantly more mutagenic than the background with S9, as was the activated mutagen, 2-amino-anthracene, with S9. All samples with S9 added were more mutagenic than all three samples without S9 (E1, E2, E3) (Table 13.5). This assay confirms the concept that liver microsomes rich in cytochrome P450 should be added to test compounds in various assays to simulate the passage of the compound or extract through the body.

Subsequent HPLC analysis (see next section) revealed that active constituents in the dried root capsule extracts (E3) were probably caffeic acid derivatives. Definite mutagenesis was detected when the *Echinacea* products were activated with S9, which is contrary to results observed by Rao

TABLE 13.5
Results Observed in Muta-ChromoPlate Assay for *Echinacea* Products (+) or (–) S9

Sample	% Positive Wells
Sterility control	0
Background + water + bacteria	23.6
Background + water + bacteria + S9[a]	62.5
2AA[b] + S9	100
Ethanolic extract	19.4
Glycerol extract	23.6
Dried root complex	19.4
Ethanolic extract + S9	100
Glycerol extract + S9	100
Dried root complex + S9	100

[a] Human liver microsome fraction S9.

[b] 2-Amino-anthracene requires activation with addition of S9.

et al. (1992) in their work with caffeic acid derivatives from propolis (bee pollen). These authors' results indicated that the caffeic acids, methyl caffeate, phenylethel cafeate, and phenylethel dimethylcaffeate, were not mutagenic with either *Salmonella* strain TA 98 or strain TA 100. They measured orinthine decarboxylase activity and protein tyrosine kinase activity of a human colon adenocarcinoma cell line when exposed to these same chemicals. This led them to conclude that caffeic acid esters present in propolis exhibited chemopreventive properties (Rao et al., 1992). Czeczot et al. (1990) tested the flavonoids, quercetin, rhamnetin, isohamnetin, apigenin, and luteolin in the Ames *Salmonella* assay with and without S9 activation. They found that quercetin actually increased the rate of mutagenesis when activated with S9, similar to the results reported herein which indicated a significant difference in mutation rate of all extracts upon S9 activation.

In a preliminary experiment, bottom agar plates were prepared with and without histidine. Plates with histidine were inoculated with a bacterial lawn of *S. typhimurim* strain TA 100 and incubated at 37°C for 48 hours and served as a control. Bottom agar (histidine-minus) was inoculated with a bacterial lawn of *S. typhimurim* strain TA 100. Extract-soaked discs were placed on the bacterial lawn and the extract constituents allowed to diffuse into the media. Growth of *S. typhimurim* strain TA 100 on histidine-plus plates confirmed the viability of the bacteria used in the assay. Absence of growth on histidine-minus plates with extract soaked discs confirmed that the extracts did not serve as a histidine source for *S. typhimurim* TA 100. Therefore, bacterial growth in the Muta-Chrome assay in histidine-minus wells was due to *S. typhimurim* revertants, and not to *Echinacea* extracts acting as a histidine source. These results support the use of the Muta-ChromoPlate assay as a reasonable test of mutagenicity for *Echinacea* products.

In conclusion, all samples in the Muta-ChromoPlate assay, regardless of source, without activation with S9 were no more mutagenic than the background. All extracts were mutagenic when S9 was added, but S9 alone increased the rate of mutagenesis. Even though S9 alone increased the rate of mutagenesis, the root complex extracts plus S9 were significantly more mutagenic than the background plus S9, as was the activated mutagen, 2-amino-anthracene plus S9. The results in this study revealed definite mutagenesis when activated with S9, and confirm the concept that liver microsomes should be added to test compounds in various assays to simulate the passage of the compound through the body. The increased mutation rate observed in the Muta-ChromoPlate prohibits the use of any of the products tested by carcinoma patients and serves as a precautionary note for other manufacturers' dried root complex products.

CONSTITUENT ANALYSIS

Products derived from a living system are inherently variable. *Echinacea* products are no exception. Factors contributing to the variability of plant material include environmental conditions under which the plant was grown, the plant part harvested, and perhaps the time of harvest and even the time of day that the plant was harvested. In addition to these plant variables, the constituents found in the products themselves are variable based on extraction method and solvent used, as well as length of time from harvest in the field until they are prepared, shipped, and ultimately used. Standardization is the key to quality control within the herbal products industry and high-performance liquid chromatography (HPLC) for marker compounds is one analytical method used to test product consistency. Degradation of marker compounds during extraction from *Echinacea* samples has been observed (Nusslein et al., 2000). Others have monitored the concentration of marker compounds in *Echinacea* as related to drying methods (Kim et al., 2000). Marker compounds used for quality control of *Echinacea* products consist of caffeic acid derivatives. The following study examined the HPLC profile for selected marker compounds in each of three *Echinacea* products, and the effect of human liver microsomal activation (S9) on their concentration.

Experimental Protocol

Plant samples were derived from the same source as used in the antineoplastic activity bioassays. Analysis of plant samples involved HPLC analysis after extraction and selected constituent profiles were established for chlorogenic acid, caftaric acid, cynarin, and cichoric acid. The dried root complex was incubated with S9, and the extraction and analysis repeated as a function of incubation time. A typical HPLC profile is shown in Figure 13.4.

Results and Discussion

None of the marker compounds were detected in the ethanolic tincture, and the glycerol extract (E1 and E2) showed no detectable compounds. The inability to detect any of the caffeic acid derivatives in ethanolic and glycerol extracts may be attributed to their degradation during the commercial extraction process used, as observed by Nusslein et al. (2000). These authors observed that cichoric acid, extracted from *E. purpurea*, appeared to be degraded during extraction procedures. They found that polyphenol oxidases released during extraction procedures were responsible for the degradation of caffeic acid derivatives. Their objective was to stabilize the cichoric acid of *E. purpurea* extracts. In a medium containing 40% ethanol and 50 mM ascorbic acid, Nusslein et al. (2000) were able to keep the cichoric acid content of *Echinacea* samples constant for over 4 weeks. Likewise, Facino et al. (1993) detected cichoric acid, cynarin, and caftaric acid, using fast atom bombardment tandem-mass spectrometry with *E. angustifolia* extracts.

The dried root complex (E3) showed several components upon HPLC analysis (Figure 13.4). Four major peaks were observed with retention times at approximately 5, 9, 14, and 18 minutes. Upon addition of the S9 mix, each area of these four peaks decreased indicating product degradation (Figure 13.4). Peak 1 (~5 minutes) decreased by 36% at 30 minutes, 56% at 1 hour, 75% at 2 hours, and 99.1% at 3 hours (Figure 13.5). Peak 2 (~9 minutes) appeared to increase by 21% at 30 minutes, and then was absent at 1, 2, and 3 hours (Figure 13.5). Peak 3 (~14 minutes) decreased by 70% at 30 minutes, and appeared to increase at 1 hour by 11%, while at 2 hours the peak area appeared to increase by 18% and decreased by 99.97% by 3 hours (Figure 13.6). Peak 4 (~18 minutes) decreased by 43% at 30 minutes, increased 5% over the 30-minute sample at 1 hour, decreased to 48% at 2 hours, and decreased by 74% at 3 hours (Figure 13.6). None of the marker compounds were detected in the S9 blank. Percent decrease was calculated based on the reduction in area of the peak as compared to the baseline run of E3 without S9 added.

In a subsequent analysis with *E. purpurea* standards, Peak 1 appeared to be chlorogenic acid, Peak 2 appeared to be caftaric acid, Peak 3 appeared to be cynarin, and Peak 4 appeared to be

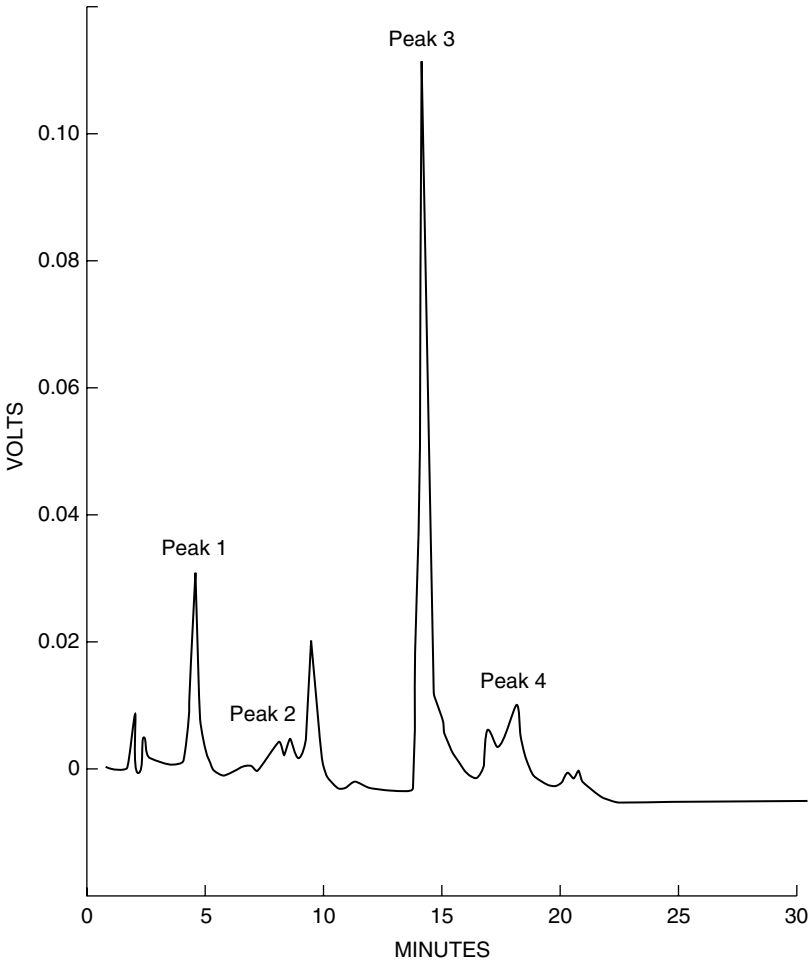
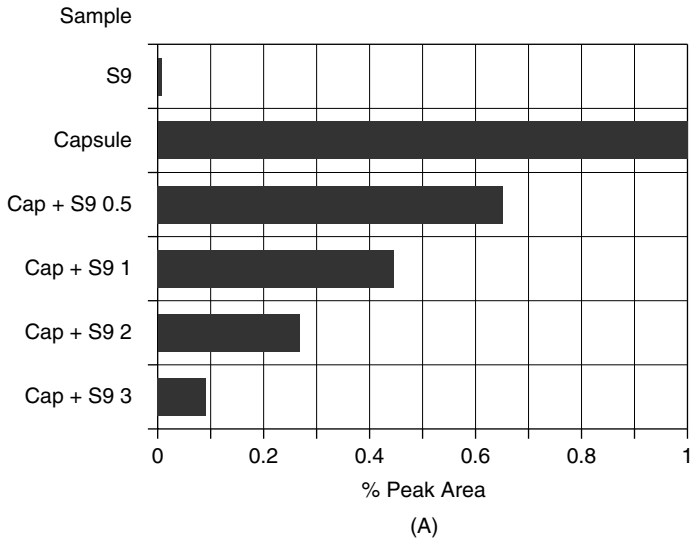


FIGURE 13.4 HPLC profile of dried root complex (tentative identifications: Peak 1, chlorogenic acid; Peak 2, caftaric acid; Peak 3, cynarin; Peak 4, cichoric acid).

cichoric acid. The initial degradation with subsequent increase in peak area for Peaks 2, 3, and 4 may be due to the breakdown of one of the other compounds represented by the other peaks on the original chromatogram. Cichoric acid and caftaric acid are caffeic acid derivatives (Figure 13.7).

As noted previously, ethanolic and glycerol extracts were derived from the whole *E. purpurea* plant, and the root complex capsule was derived from roots of *E. purpurea* and *E. angustifolia*. Perry et al. (2001) analyzed *E. purpurea* species for phenolic compounds by HPLC analysis and found that *E. purpurea* roots and flower tops both contained cichoric acid and caftaric acid. They observed cynarin only in *E. angustifolia* roots (Perry et al., 2001). The observation of cynarin in root complex samples may be due to the fact that the root complex capsule was derived from roots of *E. purpurea* and *E. angustifolia*. The absence of cynarin in glycerol and alcohol extracts may be due to the fact that no *E. angustifolia* was included in the products. The inability to detect any of the caffeic acid derivatives in glycerol and ethanolic extracts may be due to inaccurate identification of raw materials or the extraction process used for sample preparation, as well as degradation of the components over time. This enforces the notion that herbal preparations must be standardized.



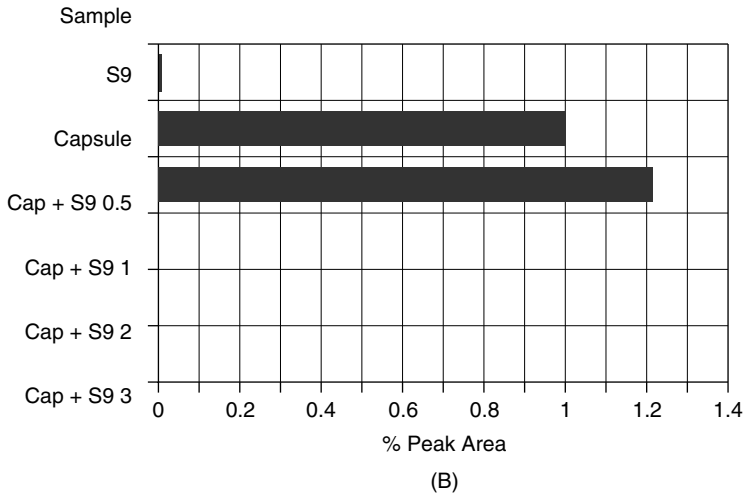
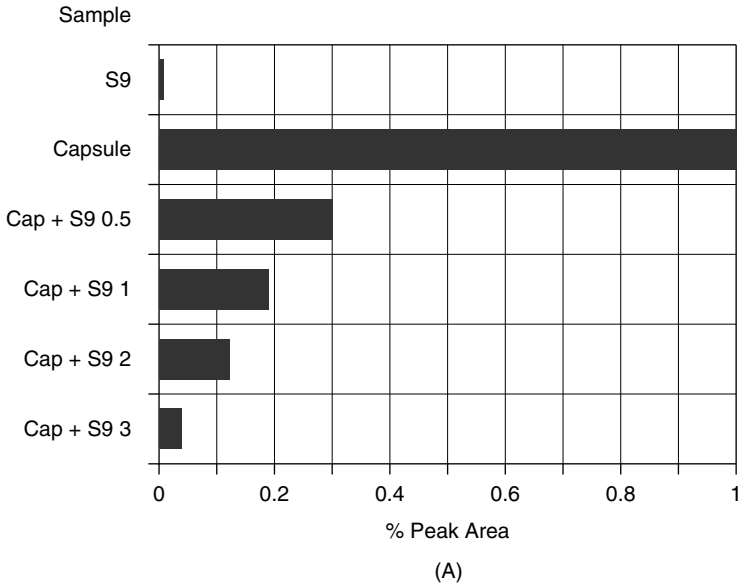


FIGURE 13.5 HPLC analysis showing degradation of peaks 1(A) and 2(B) upon addition of human liver microsome fraction S9. Samples tested were the S9 alone, dried root capsule alone, and dried root capsule + S9 at incubation times of 0.5, 1, 2, and 3 hours.

CONCLUSIONS

Echinacea products stimulate cell production and are used in pharmaceutical preparations in Europe to treat both viral and bacterial respiratory illnesses, wound infections, and other infirmities. They are also used by cancer patients to increase production of white blood cells after therapeutic treatments. Although some patients may prepare their own extracts, the majority of patients use commercial products. The study reported here was performed on products purchased from a local store in three different forms — ethanolic tincture, glycerol extract, and dried root complex capsules — and incorporated the addition of S9 microsomal fractions from human liver in bioassays. Our objectives were to determine: (1) the effect of *Echinacea* products on tumor inhibition, using a



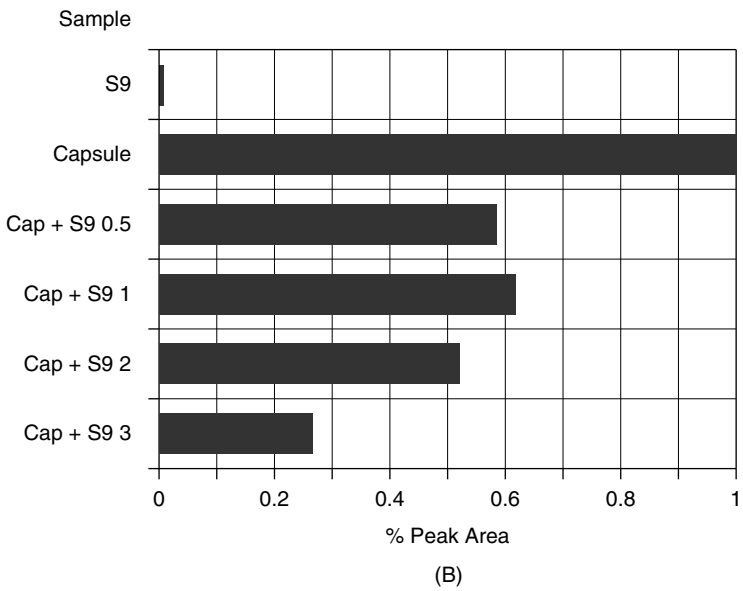


FIGURE 13.6 HPLC analysis showing degradation of peaks 3 (A) and 4 (B) upon addition of human liver microsome fraction S9. Samples tested were the S9 mixture alone, dried root capsule alone, and dried root capsule + S9 at incubation times of 0.5, 1, 2, and 3 hours.

potato tumor induction assay; (2) the effect of *Echinacea* products on the metabolic activity of tumor cells in culture, using a dimethylthiazole cell-proliferation assay; (3) the effect of *Echinacea* products on mutation rates, using the Muta-ChromoPlate assay; and (4) constituent profiles of *Echinacea* products as a function of time and microsomal enzyme activation, using HPLC analysis. Results obtained in this study are summarized below and in Table 13.6.

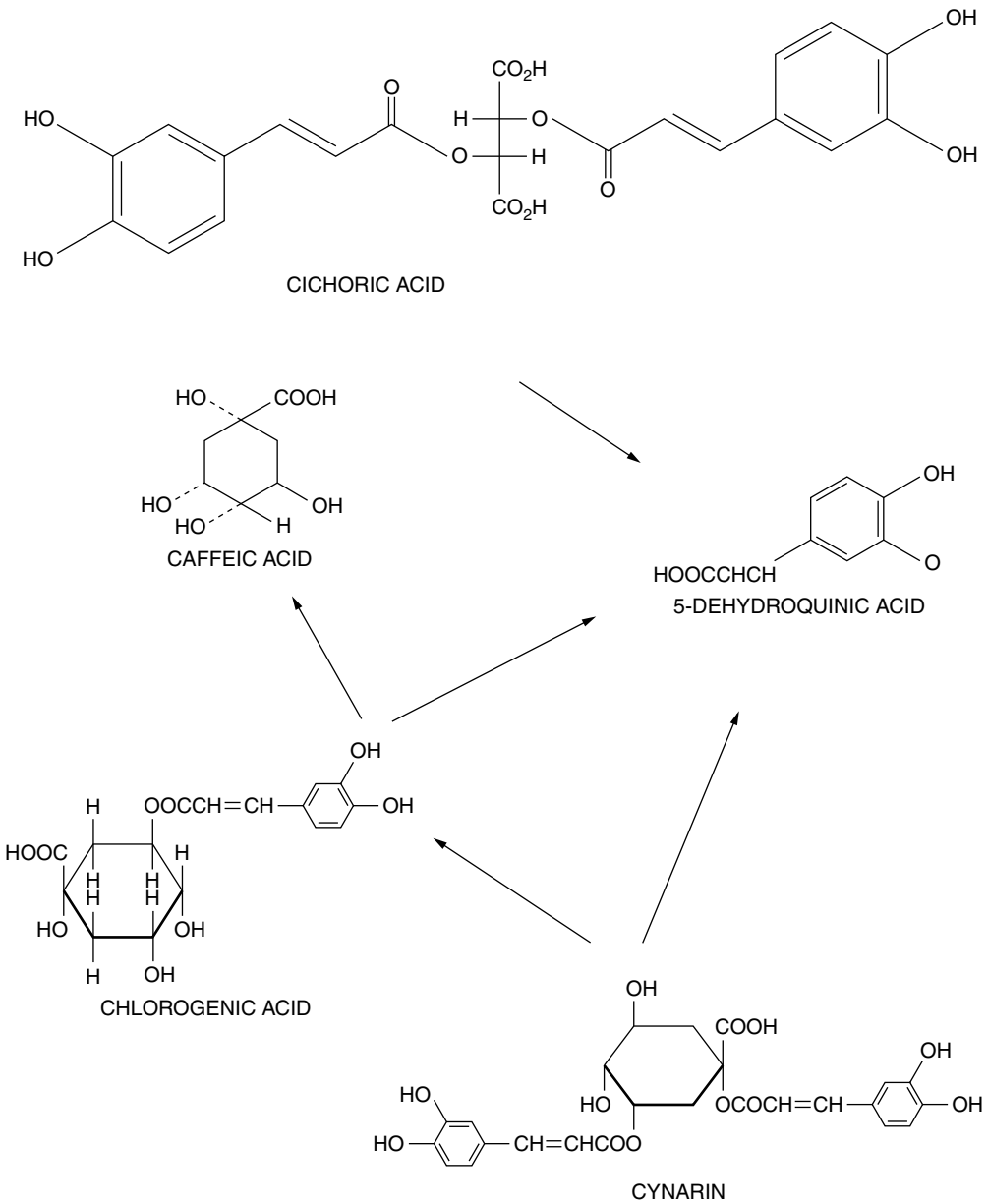


FIGURE 13.7 Suggested degradation pattern for marker compounds observed in *Echinacea* products.

Ethanolic Tinctures

Ethanolic tinctures were not inhibitory in the potato tumor induction assay.

- Upon activation with human S9, both cervical cancer and breast cancer cells exhibited an increase in metabolic activity when exposed to ethanolic tinctures.
- Upon S9 activation, ethanolic tinctures were found to be mutagenic in the Muta-ChromoPlate assay.

TABLE 13.6
Summary Table of Bioassay Results

Assay	Ethanolic Tincture	Glycerol Extract	Dried Root Complex
PTA	Negative	Inhibitory	Inhibitory
MTS + SiHa	Increased activity	No effect	No effect
MTS + MCF7	Increased activity	Increased activity	Increased activity
MUTA	Mutagenic	Mutagenic	Mutagenic
HPLC	Below detectable limits	Below detectable limits	Four major peaks detected: chlorogenic acid, caftaric acid, cynarin, cichoric acid

PTA = potato tumor induction assay; MTS = dimethylthiazole *in vitro* assay; MUTA = Muta-ChromoPlate assay; HPLC = high-performance liquid chromatography.

Glycerol Extract

- Glycerol extracts were inhibitory in the potato tumor inhibition assay.
- Upon S9 activation, glycerol extracts had no effect on cervical cancer cells, but increased the metabolic activity of breast cancer cells.
- Upon S9 activation, glycerol extracts were found to be mutagenic in the Muta-ChromoPlate assay.

Dried Root Complex

- Dried root complex samples were inhibitory in the potato tumor inhibition assay.
- Upon S9 activation, dried root complex samples had no effect on cervical cancer cells, but increased the metabolic activity of breast cancer cells.
- Upon S9 activation, dried root complex samples were found to be mutagenic in the Muta-ChromoPlate assay.
- Marker compound analysis showed four major peaks that appeared to be chlorogenic acid, caftaric acid, cynarin, and cichoric acid.
- Upon addition of S9 mix, each of these four peaks was initially degraded, but then caftaric acid, cynarin, and cichoric acid appeared to increase as degradation time was increased. Because cichoric acid and caftaric acid are caffeic acid derivatives, the initial degradation with subsequent increase in peak may have been due to the breakdown of earlier detected peaks.

These results appear to prohibit the use of those *Echinacea* products analyzed, by carcinoma patients, and serves as a precautionary note for other manufacturers' products. Furthermore, the results confirm the notion that these products require stringent standardization and quality control before they may be considered safe. Only one brand of each formulation was tested in this study, as well as only one breast cancer cell line and one cervical cell line. The variability of these results illustrates the problem of standardization within the herbal preparations industry, and within formulations and brands, as well as the variability within plants. Constituents vary from plant to plant according to environmental conditions, time of harvest, and portion of the plant harvested. In addition to this inherent inter- and intra-plant variability, manufactured products differ according to extract procedures, solvents used, and shelf life.

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Section VI

Clinical Assessment of the Medicinal Use of Echinacea *Species Products: Positive Effects and Contraindications*

14 *Echinacea*: Quality, Uses, and Immunomodulating Activity from a Phytotherapist's Perspective

Kerry Bone

CONTENTS

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INTRODUCTION

Echinacea is probably the most widely used herbal product in the English-speaking world. However, despite its popularity, the scientific understanding of how *Echinacea* works on the immune system is incomplete. The scientific information that does exist has sometimes been overenthusiastically applied or even misinterpreted. Unfortunately, this has led to some writers suggesting restrictions and contraindications for the use of *Echinacea* that are premature at best and probably ill advised.

Some of these limitations are essentially derived from the concept that *Echinacea* stimulates the immune system. The following assumptions are then made:

Since it is not healthy to stimulate the immune system all of the time, *Echinacea* should only be used as a short-term treatment.

Stimulation of the immune system will be detrimental in autoimmune disorders (such as multiple sclerosis) or in disorders where a heightened immune response may be counterproductive (such as AIDS, asthma, leukemia, and tuberculosis); hence *Echinacea* is contraindicated in their treatment (Blumenthal et al., 1998).

Underpinning these hypothetical limitations on *Echinacea* use is a model of its pharmacological activity that is largely based on extrapolation from test tube research. This article will clarify in particular the relevance of polysaccharide research, and propose, on the basis of traditional use and modern research, that several popular restrictions placed on the use of *Echinacea* are unfounded and possibly counterproductive.

Interpretation of the research on *Echinacea* is also complicated by the phytochemical variability across different preparations of this herb. The implications of this issue will also be highlighted.

WHAT IS *ECHINACEA*?

A fundamental complicating factor in interpreting the research on *Echinacea* is that the name of the genus is used to describe many different preparations in use around the world. These include:

1. The stabilized juice of *E. purpurea* tops, which is often sold under the trade name Echinacin.
2. Fresh or dried whole plant or aerial preparations of *E. purpurea, Echinacea angustifolia*, or *Echinacea pallida*.
3. Fresh or dried preparations from the roots of *E. purpurea, E. angustifolia*, or *E. pallida*.
4. Mixtures of any of the above.

Preparations of the above are given in various dosage forms including tablets, liquids (in ethanol-water mixtures or other), capsules, and dried extracts (in tablets or capsules). Some preparations are administered by intramuscular injection in some countries, especially in Europe. It would be unreasonable to expect that all of these preparations and dosage forms are likely to contain the same chemical profile and have the same pharmacological effects in the human body. The need for greater clarification of the phytochemical content of *Echinacea* preparations used in clinical studies was highlighted by Dennehy (2001).

Excluding the situation in Germany, most phytotherapists do not use *E. purpurea* stabilized juice by injection, and yet the research on this product and dosage form comprises much of the clinical work on *Echinacea*. In short, much of the research on *Echinacea* is probably irrelevant to the ways in which it is commonly used by phytotherapists in the English-speaking world, that is, oral preparations, and particularly from the roots of *E. angustifolia* and/or *E. purpurea*.

WHAT MAKES *ECHINACEA* WORK?

The possible active components that may occur in the various *Echinacea* preparations can be divided into three major groups: caffeic acid derivatives; polysaccharides; and lipophilic components, most notably the alkylamides (or alkamides) in the case of *E. purpurea* and *E. angustifolia.*

Caffeic Acid Derivatives

Echinacoside has weak antibacterial activity that is probably insignificant for the normal use of *E. angustifolia* and *E. pallida* (Stoll et al., 1950). What is more significant is that echinacoside has not exhibited immunological activity in any test applied (Bauer and Wagner, 1991). Therefore, for extracts that are standardized to echinacoside, this entity merely acts as a marker compound, rather than as an indicator of immune-enhancing activity. Since echinacoside is not unique to a single *Echinacea* species, and since the temptation to optimize extraction to give the highest yield of what is essentially an inactive compound will always exist, it is suggested that other markers of identity and/or activity should be chosen.

In contrast to the lack of activity of echinacoside, chicoric acid from *Echinacea* caused a marked stimulation of phagocytosis in an *in vitro* granulocyte test, which was confirmed in an *in vivo*

carbon-clearance test (Bauer et al., 1989). This suggests that chicoric acid may be an important active component, mainly in the *E. purpurea* root and herb.

Polysaccharides

Much of the confusion about *Echinacea* has arisen from misinterpretation or overemphasis of the polysaccharide research. Statements such as: "*Echinacea* will not be immunologically active if given as an ethanolic extract," or "*Echinacea* is a T-cell activator," or "*Echinacea* is contraindicated in AIDS," have all arisen from an overly enthusiastic interpretation of the pharmacological literature pertaining to *Echinacea* polysaccharides.

Early studies on a crude polysaccharide mixture from the aerial parts of *E. purpurea* showed that this mixture stimulated T-lymphocyte numbers and activity *in vitro* (Wagner and Proksch, 1981). This mitogenic action on T-lymphocytes was probably due to nitrogenous impurities in the polysaccharide mixture, since this activity was found to be extremely low in later investigations with purified polysaccharides (Bauer and Wagner, 1991). These nitrogenous (protein) impurities are unlikely to survive normal human digestion. Nonetheless, the herbal literature still abounds with statements that *Echinacea* enhances T-cell function, often with elaborate pharmacological theories based on polysaccharide activity.

Echinacea polysaccharides (EPS; a protein-free, highly enriched polysaccharide mixture from the aerial parts of *E. purpurea*) seem to preferentially stimulate the mononuclear immune system *in vitro* (Bauer and Wagner, 1991). EPS stimulated both peritoneal and bone marrow macrophages to behave cytotoxically *in vitro*. In a second experiment it was shown that EPS stimulated bone marrow macrophages to release interleukin 1 (IL-1), although it was much less potent than endotoxin in this respect (Bauer and Wagner, 1991).

Subsequent research was mainly on an acid arabinogalactan (AG) or an industrially prepared polysaccharide mixture (EPAG) that differs markedly from those found in *E. purpurea*, both isolated from tissue cultures of *E. purpurea* (Bauer and Wagner, 1991). (Tissue cultures do not necessarily represent what is found in the living plant.) AG induces a dose-dependent release of tumor necrosis factor α (TNF-α) from peritoneal macrophages *in vitro*. When bone marrow macrophages were used *in vitro*, a dose-dependent release of interferon-β_2 (IL-6) was also found. There is also indirect evidence that EPAG stimulates TNF-α from peritoneal macrophages *in vitro*. The effect of *Echinacea* arabinogalactan from tissue cultures (AG or EPAG) is strikingly selective for macrophages *in vitro* (Bauer and Wagner 1991). Juice from *E. purpurea* leaf also has these properties *in vitro* (Burger et al., 1997).

EPAG given by intravenous injection to mice at the very high dose (relative to levels in *Echinacea*) of 10 mg/kg resulted in a protective effect against *Candida albicans* infection (Lohmann-Matthes and Wagner, 1989). Other similar tests have been performed with positive results.

The problem with attempts to explain the activity of the traditional preparations of *Echinacea* (especially ethanol-water extracts of *E. purpurea* or *E. angustifolia* root) in terms of polysaccharides, apart from the fact that polysaccharides are not very soluble in ethanol, is the uncertain bioavailability of these compounds. This issue has been highlighted in a recent clinical trial on *Echinacea* polysaccharides, where they were administered by injection to optimize bioavailability. The trial demonstrated only modest results on immune function (Melchart et al., 2002). In this open prospective study with matched historical controls, a polysaccharide fraction isolated from *E. purpurea* cell cultures was tested to see if it could counter the undesired side effects of cancer chemotherapy. Fifteen patients with advanced gastric cancer undergoing palliative chemotherapy with a range of cytotoxic drugs also received daily intravenous injections of 2 mg of a polysaccharide fraction from *Echinacea*. While the polysaccharide treatment did appear to increase white cell counts, there were no clinically relevant effects on phagocytic activity or lymphocyte subpopulations. Melchart et al. (2002) suggested that this form of treatment should be investigated further.

Lipophilic Components

In landmark research on *Echinacea*, Bauer et al. (1988) demonstrated considerable immunological activity for the lipophilic components of the roots of the three major species of *Echinacea*. As with other research on *Echinacea*, this work has sometimes been misinterpreted. The roots were first extracted with pure ethanol (which would exclude polysaccharides). Then a lipophilic fraction (chloroform fraction) and a polar fraction (water-soluble fraction) were separated from this original ethanolic extract. The three solutions were then tested for each species using two pharmacological tests — the carbon-clearance test after oral administration and the granulocyte smear test, which is an *in vitro* test.

Results showed significant enhancement of phagocytosis for the following (Bauer et al., 1988):

- In the carbon-clearance test, for ethanolic extracts of *E. purpurea*, *E. angustifolia*, and *E. pallida*. For *E. pallida*, the chloroform and water-soluble fractions were also tested, but only the chloroform fraction was active. For *E. purpurea*, only the water-soluble fraction was tested, and it was found to be active, although this activity was less than the ethanol extract. Fractions of *E. angustifolia* were not tested.
- In the granulocyte smear test it was found that all the ethanolic root extracts caused *in vitro* a 20% to 30% increase of phagocytosis. Chloroform fractions of *E. pallida* and *E. angustifolia* were considerably more active than their water-soluble fractions, which showed negligible activity. In contrast, high activity was found in both the chloroform and water-soluble fractions of the ethanolic extract of *E. purpurea*.

Bauer et al. (1988) analyzed the various fractions tested in the study. The chloroform fractions mainly contained alkamides for *E. purpurea* and *E. angustifolia* and polyynes for *E. pallida*. The water-soluble fractions contained the characteristic caffeic acid derivatives of each root. Based on their research, these compounds are important for the activity of *Echinacea* root, not the polysaccharides.

One misunderstanding that has resulted from this work is that the water-soluble fractions of *Echinacea* roots have significant immunological activity. This has led to an argument for low ethanol extracts of *Echinacea* root. But what was really tested by Bauer et al. (1988) was the water-soluble fraction of a pure ethanol extract. So their work, in fact, supports the value of high-percentage ethanol extracts of the root. (The Eclectics of the late 19th and early 20th centuries used an 80% ethanol extract of *E. angustifolia* root.)

Recently Bauer and co-workers (Dietz et al., 2001) found that the lipophilic (and therefore ethanol-soluble) alkamides could be detected in the bloodstream after oral doses of *Echinacea*, thus establishing their bioavailability. Also, the immune-stimulating activity of *Echinacea* alkamides has been demonstrated in pharmacological models (Goel et al., 2002). Anyone wishing to explain the activity of traditional preparations of *Echinacea* should be looking in this direction.

QUALITY ISSUES WITH *ECHINACEA* PRODUCTS

The variability of the phytochemical content of *Echinacea* products was highlighted by a survey of *Echinacea* products on the German market, which found wide variations in levels of tested phytochemical components (Osowski et al., 2000). The authors chose to use chicoric acid and the two main alkamides in *E. purpurea* as quality markers, since these have been described as immunomodulating active components in this species. The same alkamides are also found in *E. angustifolia*. In regard to these quality markers, the authors found highly concentrated products as well as those without any detectable chicoric acid or alkamides. The concentration of both marker compounds varied markedly depending on how the product was manufactured (homeopathic mother tincture, pressed juice, tablets, spagyric tincture) and the species and plant part used. Even more

disturbingly, large differences in quality were found among different batches of the same product. Osowski et al. (2000) recommended that any pharmacological or clinical studies with *Echinacea* products should always include quantification of the potentially active components.

Not long after this publication, ConsumerLab.com, an independent evaluator of dietary supplements, released the results of its product review of *Echinacea* products (ConsumerLab.com, 2001). Eleven of 25 *Echinacea* products available on the U.S. market (i.e., 44 per cent) did not pass the criteria set by ConsumerLab.com for its quality review. Six products did not provide sufficient label information to identify the amount and form of *Echinacea* used or the species or plant part used (a Food and Drug Administration requirement), and were dropped from further testing. Four products had insufficient levels of marker compounds and one product exceeded the World Health Organization limit for microbial contamination.

The results of these two surveys are supported by similar results from a quality evaluation of *E. purpurea* products on the Australian market (Wills and Stuart, 1998). All three studies support what many phytotherapists have maintained for some time: the efficacy of *Echinacea* products varies tremendously due to the quality of raw material, the plant part and plant species used, and the manufacturing process. This variability makes it difficult to provide a meaningful assessment of the current pharmacological and clinical data for *Echinacea* (Melchart and Linde, 1999).

Recent studies have provided some insight into the observed variability in marker compounds in *Echinacea* products. Apart from variation due to plant part (the roots of *E. purpurea* contain higher levels of alkamides and lower levels of chicoric acid compared to the leaves), different methods of drying can dramatically alter the profile of marker compounds (Kim et al., 2000a, 2000b). Using a fresh plant extract is not the answer because enzymatic degradation will destroy the chicoric acid and the highly lipophilic alkamides will not be effectively extracted (Nusslein et al., 2000).

Regarding the two recent surveys, the levels of alkamides found in the German products (either leaf or root) were nowhere near the levels found in high-quality root extracts. The highest alkylamide level was 0.06 mg/ml whereas a good-quality 1:2 extract of *E. purpurea* root could contain more than 10 times this level (Lehmann, 2002). The ConsumerLab.com study unfortunately did not use alkamides in its quality assessment and it would have been interesting to see how many products passed under that (more stringent) assessment.

LIMITATIONS ON THE USE OF *ECHINACEA*

As outlined at the beginning of this chapter, monographs on *Echinacea* and anecdotal accounts often refer to limitations on its use. In particular, there is referral to the concept that *Echinacea* will cause a tachyphylaxis in immune response and hence should only be used for 5 days or so. Certainly continuous use is not advised by several sources. Also it is often written that *Echinacea* is contraindicated in autoimmune disease. The origin of this highly cautious approach to what is a relatively benign agent needs to be critically examined.

Traditional Use Does Not Support Limitations

The concept of traditional use is very misunderstood. For example, conventional medical scientists often confuse traditional use information with that from folk use or anecdotal accounts. It is important that the concept of traditional use is elevated to the high status it deserves.

In the case of *Echinacea*, information about its use first came from Native American tribes. Their use of *Echinacea* was then adopted by the Eclectics, a group of practitioners who were prominent around the late 19th and early 20th centuries in the U.S. By 1921, *Echinacea* (specifically the root of *E. angustifolia*) was by far the most popular treatment prescribed by Eclectic physicians (Wagner, 1996). The Eclectics used *Echinacea* for about 50 years, which is a relatively short period in the context of traditional use. However, given that the Eclectic use of *Echinacea* was based on

tribal knowledge and that they accumulated extensive clinical experience in its use, their traditional use data is of a relatively high quality. The best sources of these data are *King's American Dispensatory* (Felter and Lloyd, 1993) and Ellingwood (1993).

The extensive range of conditions for which the Eclectics prescribed *Echinacea* is summarized in Table 14.1. It is clear from this table that the limitations on *Echinacea* suggested by modern writers are not supported. The conditions in the table are mainly infections and envenomations of various kinds (which clearly attest to *Echinacea*'s influence on the immune system). However, the inclusion of tuberculosis and disorders related to autoimmunity such as diabetes, exophthalmic goiter, psoriasis, and renal hemorrhage contrasts with the contraindications suggested by some modern writers.

TABLE 14.1
Eclectic Uses of *Echinacea*

Abscesses	Mastitis, acute and chronic
Alopecia	Measles
Anthrax	Meningitis
Appendicitis	Nasal catarrh
Bed sores	Psoriasis
Bee sting	Puerperal infection
Boils	Pulmonary gangrene
Cancer	Purulent salpingitis
Carbuncles	Quinsy
Chicken-pox	Rabies
Cholera	Renal hemorrhage
Chronic bronchitis	Respiratory catarrh
Chronic glandular indurations	Scarlet fever
Chronic malaria	Scorpion sting
Chronic ulcerations	Septic injuries
Diabetes mellitus	Septicemia
Diphtheria	Small pox
Dysentery	Snake bite
Eczema	Spider bite
Empysema	Syphilis and syphilitic nodules
Epidemic influenza	Tetanus
Erysipelas	Tonsillitis
Exophthalmic goiter	Tubercular abscesses
Fevers	Tubercular phthisis
Gangrene	Typhoid fever
Gonorrhea	Typhoid pneumonia
Impetigo	Ulcerative stomatitis
Impotence	Urethral infection
Intestinal indigestion	Vulvitis
Leg ulcers	Wasp sting
Leucorrhea	Wounds
Malaria	

Sources: Summarized from Felter, H.W. and Lloyd, J.U., 1993, *King's American Dispensatory*, 18th ed., vol. 1, Eclectic Medical Publications, Portland, OR; and Ellingwood, F., 1993, *American Materia Medica, Therapeutics and Pharmacognosy,* vol. 2, Eclectic Medical Publications, Portland, OR.

The Eclectics were also not averse to using *Echinacea* over the long term. For example, according to Ellingwood (1993), *Echinacea* was recommended for the following chronic conditions: cancer, chronic mastitis, chronic ulceration, tubercular abscesses, chronic glandular indurations (hardening), and syphilis. With regard to syphilis, Ellingwood (1993) writes: "The longest time of all cases yet reported, needed to perfect the cure, was nine months." He cites a dramatic case history of vaccination reaction where *Echinacea* was taken every 2 hours for up to 6 weeks.

Modern Research Does Not Support Limitations

One published clinical study has been subjected to considerable overinterpretation, which has led some writers to suggest that *Echinacea* depletes the immune system when used continuously for periods longer than several days. This is the study by Jurcic et al. (1989) that tested the effect of an *E. purpurea* root tincture on the phagocytic activity of human granulocytes following intravenous or oral administration. The results from the oral dose part of this study are adapted in Figure 14.1. A cursory examination of the figure might lead to the conclusion that use of *Echinacea* for more than a few days depletes the phagocytic response. However, this would be a misinterpretation of the results. The arrows at the bottom of the figure indicate the application of the test dose, which was administered for only the first 5 days. While the *Echinacea* was given, phagocytic activity remained high. Only when *Echinacea* was stopped does the phagocytic activity decline to normal levels, a typical washout effect. The study in fact demonstrated the following:

- Phagocytic activity remains higher than normal while *Echinacea* is given.
- When *Echinacea* is stopped, phagocytic activity remains well above normal for a few days, indicating that far from causing depletion, there is a residual stimulating effect when *Echinacea* is stopped.
- Phagocytic activity then only returns to normal, that is, there is no depleting effect where activity drops to less than normal.

A number of published clinical studies on *Echinacea* do not support the suggestion that long-term use is detrimental. For example, a review of published *Echinacea* studies by Parnham (1996) found

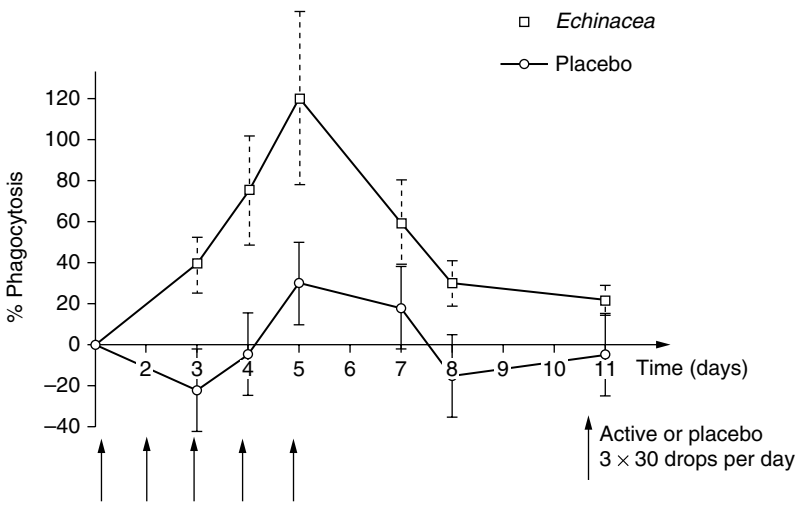

FIGURE 14.1 Oral double-blind study with *Echinacea purpurea* versus placebo. (From Jurcic et al., 1989, *Z. Phytother.*, 10: 67–70. With Permission.)

that adverse events on oral administration for up to 12 weeks are infrequent and consist mainly of digestive symptoms. Parnham (1996) concluded that *Echinacea* is well tolerated on long-term oral administration. Another study found that immune reactivity after 10 weeks of continuous oral doses of *Echinacea* was considerably greater than after 2 weeks, which in turn was significantly greater than before therapy (Coeugniet and Kühnast, 1986).

ECHINACEA IN AUTOIMMUNITY AND ASTHMA

The German Commission E monograph for *E. purpurea* herb (B Anz No 162, dated 29.08.92) states that in principle *Echinacea* should not be used in "progressive conditions" such as tuberculosis, leukemia, collagen disorders, multiple sclerosis, AIDS, HIV infection, and other autoimmune disease (Bisset, 1994, Blumenthal et al., 1998). However, the key words here are "in principle." There are no clinical studies that document an adverse effect resulting from *Echinacea* use in any of these conditions.

The suggestion that *Echinacea* is contraindicated in autoimmune disease assumes that any enhancement of any aspect of immune function is detrimental. However, immune function is extraordinarily complex and a substance that acts largely on phagocytic activity may be safe or even beneficial in autoimmunity. Many theories have been proposed as to the causative factors in autoimmune disease. However, there is growing evidence that an inappropriate response to infectious microorganisms, through phenomena such as molecular mimicry, may be at work (Bone, 1995a, 1995b). If this is the case, *Echinacea* may be beneficial in these disorders because it may decrease the chronic presence of microorganisms. There is now a body of clinical observations by phytotherapists, including mine, that long-term *Echinacea* (over months or even years) is at least not harmful in autoimmunity, and is probably beneficial in many cases.

There is concern in some circles that *Echinacea* may cause an allergic reaction in susceptible patients that may be severe or even life-threatening. This concern is also linked to the suggestion that *Echinacea* is thereby contraindicated in asthma. The Commission E monograph cautions that *Echinacea* should not be used by people who have a tendency to allergic reactions, especially against Asteraceae (Compositae; daisy family). This fear was highlighted in some sensationalist television and print media journalism in Germany in 1996 (Hansen, 1996) that attributed three deaths to *Echinacea* over a 6-year period.

A critique of these claims was written by R. Bauer, Institute for Pharmacognosy at the University of Graz, considered to be an expert on *Echinacea* (Bauer and Wagner, 1996). Bauer asserts that the health authorities saw no cause to take action on the reported cases, since a causal relationship between the deaths and the taking of *Echinacea* preparations could not be proven. For example, in the first case, the patient presented with allergic vasculitis and was dying of acute renal failure. Dr. Peter Schönhöfer (Hansen, 1996) attributed this to an allergic reaction to the plant, but he also noted that influenza can trigger a vasculitis of that type. Bauer (Bauer and Wagner, 1996) argues that for the second case, in which thrombocytopenia was connected with another *Echinacea* product, independent investigations could not establish causality. Bauer and Wagner (1996) point out that since over 10 million units of *Echinacea* products are sold annually in Germany, if the risk of allergic reaction were substantial, then more cases would have been reported. Finally, Bauer draws on his extensive research on the chemistry of *Echinacea* products, stating that any proteins that they may contain are denatured by alcohol and are unlikely to cause allergic cross-reactivity.

The previously cited review by Parnham (1996) concluded that the stabilized juice from *E. purpurea* tops (the most common form of *Echinacea* sold in Germany and the product most likely to cause allergic reaction since it includes the flowers) is well tolerated. All available published and unpublished articles containing reports of the presence or absence of adverse events were considered, provided the dose and route of administration as well as the patient population were defined. Results for several thousand patients over more than 40 years were analyzed.

An article published in the January 2002 issue of *Annals of Allergy, Asthma, and Immunology,* entitled "Adverse reactions associated with *Echinacea*: the Australian experience" (Mullins and Heddle, 2002), has reignited the debate about *Echinacea* and allergies. The authors, Raymond J. Mullins of the John James Medical Centre, Deakin, and Robert Heddle from the Flinders Medical Centre, Adelaide, discussed five patients who underwent skin prick testing (SPT) at their clinic following adverse reactions to *Echinacea*. The authors also reviewed 51 reports involving *Echinacea* received by the Australian Adverse Drug Reactions Advisory Committee (ADRAC) between January 1979 and March 2000. According to Mullins and Heddle, 26 of these ADRAC reports appear to have involved an allergic response.

As mentioned above, plants from the Compositae or daisy family (to which *Echinacea* belongs) are renowned for their allergenicity, usually due to pollen proteins. It is therefore not unreasonable to suggest that some atopic individuals may develop an allergy to *Echinacea* pollen. There have been reports of allergic reactions to *Echinacea* on the first exposure, which suggests that cross-reactivity between *Echinacea* pollen and pollen from other plants such as ragweed may have occurred.

Due to the presence of pollen, allergy is more likely to occur with products manufactured from dried flowering aerial parts. It is unlikely that an allergic response would occur with products containing the root of *Echinacea* species, since there is no pollen in this part of the plant. Considering the fact that proteins are very poorly extracted in ethanol-water mixtures it is also unlikely that an allergy would result from the fluid extracts and tinctures of *Echinacea* used by phytotherapists, even if aerial parts were used. However, it would be prudent to only use *Echinacea* root products in atopic individuals.

Unfortunately, most of the ADRAC reports lack information about the species and plant part used. One possible reason for this is the lack of understanding among the medical fraternity about the importance of this type of information. In the majority of cases, even the dosage taken by the patient is not included. In view of the limited information contained in many of these reports it is difficult to assess the role that *Echinacea* may have played in the reaction, let alone whether a particular species or plant part is most commonly responsible. According to ADRAC, there has been only one case report of anaphylaxis in association with *Echinacea* for the period November 1972 to January 2002 (the case cited by Mullins).

As with the ADRAC reports, in the five cases reported by Mullins from his own clinic, no details about dosage, species, or plant were stated. However, in answer to a communication, Mullins replied that in one of the cases a tablet consisting of 250 mg of *E. angustifolia* root powder and extracts equivalent to 1.5 g of dry *E. purpurea* herb flowering tops had been taken. In a previous case reported by Mullins (1998) in which a 37-year-old woman suffered anaphylaxis, the product also contained a combination of root and whole plant from *E. purpurea* and *E. angustifolia*.

The evidence for a link between *Echinacea* and allergy is far from conclusive but one fact is certain: considering the extensive use of *Echinacea* and *Echinacea*-containing products, much of it self-prescribed with little or no professional supervision, and even allowing for underreporting, there have been relatively few reported cases of allergy. As with any ingested substance, there is no doubt that allergic reactions to *Echinacea* will occur in a few susceptible individuals. Based on current information, these reactions are rare and are most likely to occur in preparations containing whole plant and are unlikely to occur with the *Echinacea* root products preferred by phytotherapists.

ECHINACEA AS AN IMMUNOMODULATOR

When the clinical and *in vivo* studies of *Echinacea* are carefully examined, most conclude that the herb increases phagocytic activity. Even the polysaccharides only enhance macrophage activity and killing (Bauer and Wagner, 1991). Phagocytic cells are part of nonspecific immunity. What is often not appreciated is that the activities of phagocytic cells, especially macrophages, are a key element of immune surveillance. The macrophage secretes several immune-enhancing cytokines and pro-

cesses antigenic material and then presents this to the helper T-cell. Helper T-cells can only effectively respond to antigen presented in this way. Hence, if an herb such as *Echinacea* significantly increases phagocytic activity, the end result will be enhanced immune surveillance. For infections in general, the fact that *Echinacea* increases phagocytic activity emphasizes that it works best as a prophylactic or in the very early stages of an infection. This is because enhanced phagocytosis gives better direct clearance and inactivation of pathogenic organisms by phagocytes, which is one of the first lines of immune defense, and better immune surveillance, which accelerates the response of the immune system to the new pathogen or to other opportunistic pathogens.

That *Echinacea* works best as a preventative is consistent with the clinical experience of many phytotherapists, although this activity has not been borne out by recent studies (Melchart and Linde, 1999). In fact, it may be more accurate to describe *Echinacea* as an immunomodulator. While it stimulates phagocytic activity, this may have the end result of modulating immune function overall. For example, the chronic presence of a microorganism may cause a state of immune dysregulation that results in autoimmune disease or a chronic inflammatory condition such as asthma. Such theories have been proposed in the mainstream scientific literature (Bone, 1999). A substance that enhances immune surveillance may help the body to eliminate the organism or neutralize its imbalancing effect on the immune system, thereby toning down an inappropriate immune response. Similarly, the body's response to an allergen may be reduced if a more appropriate response results from enhanced phagocytic activity and immune recognition.

CONCLUSIONS

Limitations on the oral use of *Echinacea* have resulted from preconceived and simplistic concepts of the immune system and *Echinacea*'s influence on it. Misinterpretations or overinterpretations in the scientific literature have confounded the problem. Confounding this issue further are the observations that the term *Echinacea* is used to describe many different preparations and the phytochemical content of *Echinacea* products varies considerably. However, the weight of evidence, including traditional, observational, and scientific, is that limitations on the use of *Echinacea* are ill advised.

Perhaps if the understanding of *Echinacea*'s activity after oral doses were shifted toward the concept of an immunomodulator rather than an immunostimulant, concerns about its use would abate. *Echinacea* is undoubtedly one of the most valuable herbs in use in the world today. Misconceptions about its use can only devalue its role in modern health care and needlessly restrict the efficacy of herbal therapy.

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15 The Use of *Echinacea* in Pregnancy and Lactation: A Critical Review

Moumita Sarkar and Gideon Koren

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INTRODUCTION

Complementary and alternative medicine (CAM) is an umbrella term that covers a number of health-care modalities that generally fall outside the realm of the conventional medical model (Smith et al., 1996). Herbal medicine is considered to be a primary complementary and alternative therapy. In recent years, the use of herbal products has increased dramatically, particularly in developed countries, by people who wish to maintain good health and reduce the need for conventional drug therapy.

Echinacea products are among the most popular phytomedicines. While these remedies have a long history of use in pregnancy, during delivery, and for lactation, clinically relevant sources of information on the safety and risk of such products are lacking (Lepik, 1997). Given the great variation in product composition and constituent concentration, the actual safety of *Echinacea* has not been easy to study in pregnancy and lactation. To date, there is only one published study that has examined the safety of *Echinacea* use during pregnancy for upper respiratory tract ailments (Gallo et al., 2000).

PREGNANCY FACTS

There is an underlying baseline risk for malformations associated with every pregnancy, regardless of the mother's exposure to a substance of concern. As a result, the primary objective of most

studies done in pregnancy is to determine whether pregnancy outcome is associated with any increased risk for toxicity or malformations above this baseline.

Malformations are defined as defects in organ structure or function that can vary in severity, with the most severe being life-threatening or requiring major surgery (Koren et al., 1998). On the other hand, certain drugs are considered to be toxic rather than teratogenic. This includes substances that do not cause birth defects per se, but that can damage the fetus as a result of long-term exposure during pregnancy.

PREGNANCY INDICATIONS

Over the years, *Echinacea* has become one of the most popular herbal remedies in pregnancy primarily due to its medical indications. Used both systematically and topically, it has been reported to improve the body's defenses against viral and bacterial infections, as well as to prevent and treat common cold/flu season illnesses, all of which are very common ailments during pregnancy (Melchart et al., 1994; Hoheisel et al., 1997; Grimm and Muller, 1999). The three major groups of constituents among several responsible for these effects are alkyl amides, caffeic acid derivatives, and polysaccharides (Facino et al., 1995; Combest and Nemecz, 1997).

Recommendations for *Echinacea* use are most frequently obtained through nonmedical sources, including word of mouth, friends, and family members (Gallo et al., 2000). Consequently, the quality and accuracy of information provided on product type and pattern of use may not always be reliable.

Its broad range of reported medical applications appeal to many pregnant and lactating women, who often opt for this herb over manufactured drugs because they believe it to be safer. Although anecdotal evidence may support this use, sound scientific knowledge surrounding the wide array of supplement choices is lacking (Lepik, 1997; Therapeutic Research Faculty, 2000).

RISK PERCEPTION

There appears to be a common misconception among patients and some practitioners alike that the terms "safe" and "natural" are interchangeable (Boon et al., 1999). Consequently, many women are inclined to believe that natural remedies are safer than pharmaceutical drugs (O'Hara et al., 1998). This perceived safety of natural products over manufactured drugs could increase the potential for adverse effects in both the mother and her developing fetus. This is due to the fact that many women initiate treatment with supplements such as *Echinacea* without obtaining medical advice; they either self-prescribe or take the advice of others. An added potential for concern is posed by the fact that many consumers may be unaware that unlike conventional medications, herbal products such as *Echinacea* are not under enforced regulations by the Food and Drug Administration. To further complicate matters, every country differs in their regulatory laws regarding these products. For example, minimal regulation exists in the United States, given that herbal products are classified as dietary supplements (Tsui et al., 2001).

Echinacea has been reported to be the most common herb used prior to knowledge of pregnancy and continued throughout (Tsui et al., 2001). In light of the fact that it is not subject to regulations normally applied to pharmaceuticals, it is vital for women of reproductive age to exercise both common sense as well as caution with use.

TOXICITY

Possible implications of teratogenic or mutagenic effects are often suggested on the basis of *in vitro* and animal data. Although such data are certainly useful, they cannot be used to predict reproductive effects in humans because the teratogenic potential of a substance may vary considerably among species.

To date, *in vitro* studies of bacterial and mammalian cells as well as *in vivo* studies of mice have found no evidence of mutagenicity associated with *Echinacea* (Mengs et al., 1991). There are no human studies pertaining to the effect of *Echinacea* on female fertility at this time. Recent *in vitro* studies, however, suggest possible impaired male fertility associated with *Echinacea* use (Ondrizek et al., 1999a, 1999b). This research found that high concentrations of *Echinacea* added directly to semen decreased sperm movement. But it is not always possible to extrapolate results stemming from *in vitro* research to humans, especially in light of the high concentrations used.

CLINICAL EVIDENCE

There are many implications for healthcare practitioners given the growing popularity of herbal therapy, combined with the lack of awareness for potential risks associated with unregulated products. Due to the lack of evidence-based data, health professionals caring for pregnant women are often confronted with the difficult task of counseling on the risks versus benefits of using *Echinacea* during pregnancy (Lepik, 1997). A recent study compared the attitudes and practices of physicians and naturopaths with respect to herbal products in pregnancy (Einarson et al., 2000). All naturopaths surveyed asked patients about both conventional and complementary therapy use. On the contrary, only 56% of physicians surveyed asked patients about complementary therapies during routine history taking. Despite the paucity of information for herbal use during pregnancy, naturopaths are more inclined to recommend herbal products in pregnancy. However, most pregnant women are generally under the care of conventional physicians. Lack of clinical evidence concerning safety in pregnancy was reported by these physicians to be the main reason for their hesitation, not because they deem them unsafe.

In an attempt to close this gap, the Motherisk Program conducted and published the first prospective controlled study on *Echinacea* use in pregnancy (Gallo et al., 2000). The Motherisk Program is a teratogen information and counseling service that provides evidence-based data to pregnant and nursing women and their healthcare professionals on the safety/risk of exposures such as drugs, chemicals, radiation, and infectious diseases. In service since 1985, questions posed to the program over the years have mirrored changing trends in the general population. The popularity of herbal products is reflected in the visible increase in the number of inquiries regarding the effect of these remedies in pregnancy and lactation. In the past 3 years, the total number of calls to the program averaged 32,000, with approximately 5% of all calls related to herbal products, translating to more than 1,600 calls per year.

The overwhelming number of inquiries in combination with the paucity of data prompted the need to address the implications of *Echinacea* in pregnancy. While the primary objective of the study was to determine pregnancy outcome associated with *Echinacea* use, secondary endpoints looked at pattern of use. The study consisted of women who initially contacted the Motherisk Program regarding the safety of consuming *Echinacea* in pregnancy. The study cohort included 206 women exposed to this herb who were disease matched to a control group of 206 women who had subsequently decided not to use it. Results indicated that gestational use of *Echinacea* is not associated with an increased risk for malformations above the baseline risk. In addition, no significant differences were reported in pregnancy outcome, delivery method, or fetal distress. Capsules, tablets, and tinctures were the most popular of several formulations of *Echinacea angustifolia* and *Echinacea purpurea* species used by participants. About 81% of women reported *Echinacea* to be effective in improving their upper respiratory tract symptoms. Moreover, 95% rated their perception of risk for gestational use of this herb as low. This was a reflection of the general population's perception that because herbal products are natural, they are safe.

RELATED ISSUES

It is well documented that consumption of herbal medicine can result in direct adverse effects, such as allergic reactions, nausea, vomiting, and sedation (Ernst and De Smet, 1996). Most medicinal plants contain scores of active ingredients, and unlike conventional medicinal drugs, concentrations of these elements differ from one crop to the next and even within the plant itself. As with any unregulated products, *Echinacea* use during pregnancy and lactation can be of concern, especially with issues of dosage variation, contamination, incorrect labeling, and interactions with other medications (Smith et al., 1996). For this reason, it is essential for pregnant and nursing mothers to be educated about these issues.

Length of Exposure

There is much controversy surrounding the issue as to whether *Echinacea* can be used for extended periods of time. The German Commission E does not recommend continuous use of *Echinacea* beyond 8 weeks (Blumenthal et al., 2000). Theoretical concerns of hepatotoxic effects associated with long-term *Echinacea* use have been suggested, but never substantiated (Miller, 1998). Unknown implications of prolonged use prompted most women in the *Echinacea* study to limit use to a few days, as this was reportedly sufficient in alleviating the initial symptoms of a cold. Only two women reported use on a daily basis to maintain their immune system, with no resulting adverse pregnancy outcome (Gallo et al., 2000).

Check Labels

It is critical to check labels as various other products can be found in combination with *Echinacea*. For example, goldenseal is contraindicated in pregnancy. While *Echinacea* may be safe, goldenseal, which is often contained in *Echinacea* products, contains pharmacologically active alkaloids that can lead to uterine-stimulating effects (Farnsworth et al., 1975). Consequently, potential harm could be introduced to an unsuspecting pregnant woman. An added concern in purchasing *Echinacea* is the practice of substitution. Potential for product impurity and contamination through adulteration can lead to numerous complications in pregnancy.

Alcohol Content

Consumption of large amounts of alcohol-containing *Echinacea* tincture has been linked to possible theoretical risks for alcohol-related effects in the developing fetus. However, the pattern of use for *Echinacea* products is commonly on an intermittent and infrequent basis during pregnancy. The alcohol content found in the tincture form, when taken at maximal recommended dosage, will approximate to 1 to 2 mL (~1 tsp daily) (Newall et al., 1996). Given that pregnant women tend to use much lower dosage for shorter periods than generally recommended, this minimal amount of alcohol is highly unlikely to have an effect on pregnancy outcome (Gallo et al., 2000).

Interactions

The potential for herbal remedies to interact with conventional pharmacotherapy exists, as many women do not reveal their use of herbs to their physicians (Miller, 1998; Von Gruenigen et al., 2001). This may present significant concerns since many pregnant women consume *Echinacea* supplements concurrently with over-the-counter and prescription cold medications (Eisenberg et al., 1998).

LACTATION

There is currently no information regarding the transfer of *Echinacea* into human milk or its impact on the nursing infant (O'Hara et al., 1998). This herb generally consists of nontoxic components and hence, little or no toxicity is expected when taken at recommended doses (Hale, 2000). It is important to obtain *Echinacea* from a reliable source, as use of adulterated products can lead to the possibility of exposing the infant to hidden contaminants that can excrete into the breast milk (Kopec, 1999).

CONCLUSION

In view of the fact that over 50% of all pregnancies are unplanned (Skrabanek, 1992), inadvertent gestational exposure may be inevitable for women who intend to discontinue use once pregnant. Given the widespread use of herbal supplements and the lack of evidence on safety, it is critical that special populations, such as pregnant and lactating women, consult their healthcare providers before using these products. Currently, there are no known contraindications to the use of *Echinacea* preparations during pregnancy and lactation (Blumenthal et al., 2000). Nevertheless, a product cannot be assumed to be free of harmful effects purely based on anecdotal evidence or because it is derived from a natural source. The first prospective study assessing the safety of *Echinacea* use during pregnancy failed to detect any increased risk. However, due to the limited sample size of this single study, further investigation is necessary.

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16 Two Immunoenhancers Are Not Better than One

Sandra C. Miller

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INTRODUCTION

Immunostimulants, primarily targeting natural killer (NK) cells (Lersch et al., 1992; See et al., 1997; Sun et al., 1999), exist in root extracts of the plant *Echinacea purpurea*. NK cells have been well established throughout the last 20 years, as the first line of defense against developing tumors and virus-infected cells. Extracts from this plant are not only readily commercially available, but have become extremely popular recently for their reported health benefits including abatement of virus-mediated respiratory infections, assorted inflammations (cutaneous and other), tumors, and AIDS (Hill et al., 1996; Lersch et al., 1992; Roesler et al., 1991; Steinmuller et al., 1993; Stimpel et al., 1984; Tragni et al., 1985). There appears, moreover, to be no maximum dose at which this water-soluble herb is toxic *in vivo* (Lersch et al., 1992; Melchart et al., 1995; Mengs et al., 1991).

The neurohormone, melatonin (MLT), which is produced by the pineal gland almost exclusively during the hours of darkness, coordinates circadian biological rhythms (Mazzoccoli et al., 1997; Nelson and Demas, 1996). Among its many actions, MLT plays an immunoregulatory role (Guerrero and Teiter, 1992; Liebmann et al., 1997). Most of the current popularity of MLT derives from its value in resetting disrupted sleep rhythms resulting from the phenomenon of "jet lag," as well as in the correction of assorted sleep disorders (Pierpaoli and Regelson, 1995). Administration of MLT in humans, hamsters, and mice results in T-cell–mediated functional immunoenhancement in the periphery (Champney et al., 1997; Garcia-Maurino et al., 1997; Nelson and Demas, 1996; Pioli et al., 1993). We have, furthermore, demonstrated (Currier et al., 2000) that NK cells are numerically increased *in vivo* in the presence of exogenously administered MLT.

As with most herbal products and nutriceuticals, and other agents such as MLT, virtually nothing is known beyond their touted advantages of any long-term, potentially negative effects, or, more importantly, the effect of such agents when taken in combination with other phytochemicals/herbals, hormones, or pharmaceuticals. For example, to date, there exists no quantitative information of the effect on the hemopoietic or immune cell lineages when they are confronted, *in vivo*, with relatively long-term exposure to a combination of the widely used immunostimulants *Echinacea* and melatonin. A popular concept that "two is better than one," has never been formally tested with respect to *Echinacea*-derived products and melatonin, under stringent laboratory conditions. Such analyses

are seemingly imperative, especially with regard to two vital lineages of cells, i.e., those responsible for blood cell formation (hemopoiesis) and for disease defense (immunopoiesis).

The present study was undertaken to provide quantitative information on the hemopoietic and immune cell lineages in normal, healthy adult mice given in their diet combinations of *E. purpurea* and melatonin. This study has demonstrated that, indeed, two are not better than one.

MATERIALS AND METHODS

Male DBA/2 mice (Charles River Laboratories, St. Constant, QC, Canada) were used in early adulthood. All were housed under microisolator conditions, thereby removing the possibility of contamination by virtually all common mouse pathogens. Experimental mice received via their diet, for 14 days, a commercially prepared, powder extract of *E. purpurea* root (Santé Naturelle [A.G.] Ltée, La Prairie, QC, Canada), homogenized into finely ground chow such that each experimental mouse consumed 0.45 mg/day (dose/body weight adjusted from assorted anecdotal and experimental studies in rodents and humans). Melatonin (Schiff Products, Inc., Salt Lake City, UT, USA), was co-administered daily with *E. purpurea*, in powder form (0.0142 mg/mouse/day) for 14 days in the ground chow. Other mice were provided with melatonin-containing food only, while still others were fed untreated (control) chow for the same 14-day period. Thus, mice of identical strain, age, and gender, housed two per cage, were fed every evening at 6:00 P.M. with one of these three forms of chow. Since the active period for mice occurs during the dark hours (regulated as 12 hours from 6:00 P.M. to 6:00 A.M.), chow consumption is virtually complete by the onset of the light phase (6:00 A.M.). The dose of MLT per mouse selected was based on existing dose per body weight studies in other strains of adult mice (Demas and Nelson, 1998; Lissoni et al., 1998; Maestroni et al., 1994a) and, moreover, it compares favorably with doses commercially suggested for humans. Intercage variation in food consumption among the experimental diet cages (MLT + *E. purpurea*, or MLT only), or among the control-diet cages was ascertained and found to be insignificant or undetectable at the end of each 24-hour feeding period. With respect to between-mice variations within each experimental (or control) cage, it was assumed that the long exposure time (14 days) would have canceled out any minor fluctuations in consumption of these agents by individual animals in the same cage. Moreover, all mice were identical in body weight and clinical health status at the time the study was concluded (14 days).

All experimental mice (MLT only; MLT + *E. purpurea*) and control mice (untreated chow) were killed at 1 day after the last feeding, that is, at 15 days, by cervical dislocation, and their spleens and femurs were removed. The total numbers of nucleated hemopoietic/immune cells in each spleen and both femurs (bone marrow source) were then obtained from every mouse by means of an electronic cell counter (Coulter Electronics, Hialeah, FL, USA). Subsequently, washed, clean suspensions of single hemopoietic/immune cells from the spleen and bone marrow were prepared by methods in standard use in our laboratory. Next, microscope slides, each containing a monolayer of these cells, were prepared for each spleen and both femurs from every mouse, and each slide was then stained with a tetrachrome hematologic stain to permit clear, morphological identification of every cell on each slide. Cells in all the hemopoietic and immune lineages in both organs (spleen, bone marrow), including the precursor (proliferating) and mature forms of each, were enumerated and classified. These identification techniques were established several years ago in our laboratory (Miller et al., 1978; Miller and Osmond, 1974, 1975), and continue in regular use. They provide an accurate means of recording cell lineage/sublineage fluctuations under experimental versus control conditions (Christopher et al., 1991; Currier and Miller, 1998; Miller, 1992; Miller and Kearney, 1997; Sun et al., 1999; Whyte and Miller, 1998). The subgroup of lymphocytes known as NK cells was specifically identified by means of our standard immunolabeling techniques (Christopher et al., 1991; Currier and Miller, 1998; Sun et al., 1999; Whyte and Miller, 1998). From the percentages of each of several distinct cell types (including precursor and mature forms),

identified and recorded from 2,000 total cells (hemopoietic/immune) per organ (spleen, bone marrow) per mouse, the *absolute numbers* of each of these cell types could be calculated from the already determined *total* organ cellularity. This latter value was obtained previously for each spleen and each femur by means of the electronic cell counter at the time of animal killing.

Finally, the data were analyzed statistically by means of the student's *t*-test (two-tailed). Thus, the means of experimental and control groups of mice were calculated. Via the *t*-test, the differences were ascertained between the means of the various groups: MLT versus MLT + *E. purpurea*; MLT versus control chow; and MLT + *E. purpurea* versus control chow. This was done for each of six cell subgroups, for each organ (spleen, bone marrow) for each mouse in each of the three diets.

RESULTS AND INTERPRETATIONS

Fourteen days of *in vivo* administration of MLT alone versus the control diet had no statistically significant effect on the absolute numbers of nucleated erythroid cells (precursors of blood-borne red blood cells) in the spleen (Figure 16.1a) or the bone marrow (Figure 16.1b). These cells in the spleen were the *late* erythroid precursors, that is, small, postmitotic, possessing a very dark nucleus indicative of impending pycnosis, and a cytoplasm showing clear evidence of hemoglobin. However, they were significantly *reduced* in the spleens in mice administered both MLT + *E. purpurea*, compared to either the control diet, or MLT only groups (Figure 16.1a). A strikingly different result was obtained in the bone marrow, however, with regard to cells of the erythroid lineage (Figure 16.1b). In the bone marrow, the nucleated erythroid cells, unlike the spleen, are predominantly large, proliferating, and show no cytoplasmic hemoglobin. In this organ, the absolute numbers of these nucleated cells was significantly *elevated* (Figure 16.1b) in mice receiving MLT + *E. purpurea* versus either MLT only or control vehicle diet.

Cells of the granulocytic lineage, including both mature, functional forms (Figure 16.2a and Figure 16.2b), and their proliferating precursor forms (Figure 16.3a and Figure 16.3b), in both the spleen and bone marrow, followed a very similar response pattern when mice were given both MLT and *E. purpurea* simultaneously in the diet. That is, with respect to the functionally mature cells of this lineage (residents of both the spleen and bone marrow), MLT alone had little influence on their numbers, relative to the control diet group (Figure 16.2a and Figure 16.2b). However, when mice were fed MLT + *E. purpurea*, these cells became profoundly reduced in numbers in the spleen compared to the control diet group or the MLT only group (Figure 16.2a). Similarly, in the bone marrow, mature granulocytic cells were significantly reduced relative to mice fed MLT only or the control diet (Figure 16.2b). By contrast, immature (early proliferating precursor) cells in this lineage were significantly *elevated* in absolute numbers, in both the spleen and bone marrow of mice given MLT + *E. purpurea*. These immature cells were found in numbers 28-fold and 7-fold, in the spleen and bone marrow, respectively, of the values found in mice given the control diet (Figure 16.3a and Figure 16.3b).

In this study, therefore, we found little or no influence of MLT, administered alone, on erythroid, granulocytic cells in either organ. We have previously observed, moreover, that the herb *E. purpurea*, administered in the diet by itself had no influence on the erythroid and granulocytic cell lineages, when administered for either 7 or 14 days (Sun et al., 1999). From evidence *in vitro*, MLT appears to stimulate GM-CFU indirectly, acting through MLT receptors on bone marrow stromal cells, the latter producing subsequently a variety of hemopoiesis-driving cytokines (Maestroni, 1998; Maestroni et al., 1994a, 1994b). In accord with these findings, we found in the present *in vivo* study, a significant increase in mice consuming MLT only, of proliferating precursors of the granulocytic lineage in the bone marrow, but not in the spleen (Figure 16.3b: MLT versus vehicle).

The effect of co-administration of MLT and *E. purpurea* appears to be directly correlated with the level of cell maturity within both lineages (erythroid and granulocytic). That is, mature cells are significantly reduced in number in both organs (spleen, bone marrow) while the early, prolif-

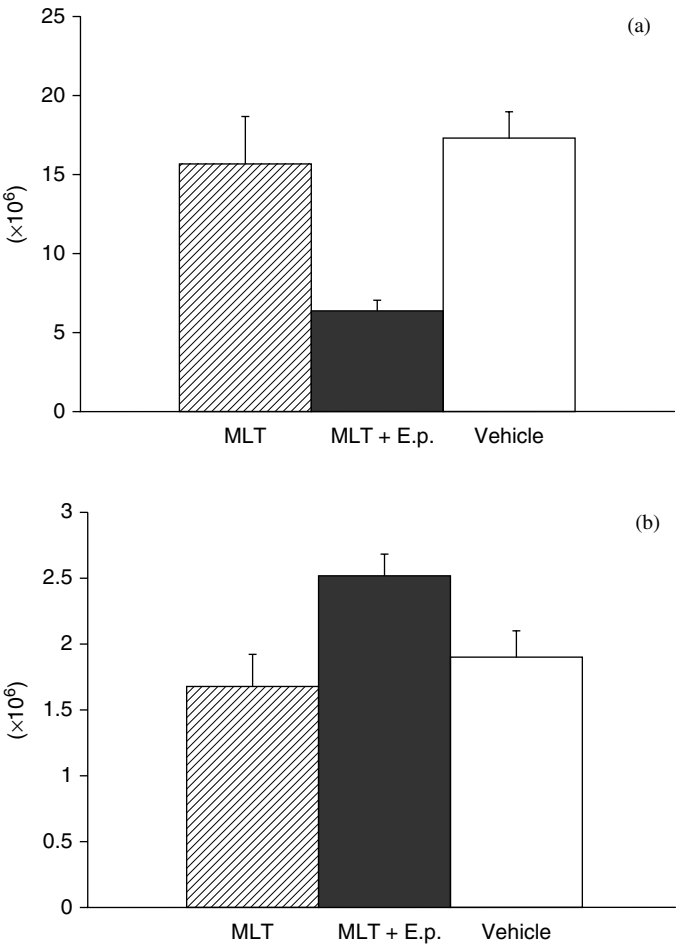

FIGURE 16.1 Absolute numbers of nucleated erythroid cells in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.02$ (MLT vs. MLT + E.p.: spleen); $p < 0.001$ (control vs. MLT + E.p.: spleen); $p < 0.02$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.04$ (control vs. MLT + E.p.: bone marrow).

erating precursors are significantly elevated in number. These observations suggest that maturation has been inhibited in the presence of both agents administered *together*, but not separately (Currier et al., 2000; Sun et al., 1999). An anti-apoptotic activity has already been ascribed to MLT (Maestroni, 1998; Provinciali et al., 1996; Yu et al., 2000), and it is possible that this effect is enhanced, additively or synergistically, by the presence of some as yet unidentified component in *E. purpurea*, resulting in the observed accumulation of the early erythroid- and granulocytic-proliferating precursors, concomitant with a striking paucity of their mature progeny. *E. purpurea*, however, when administered alone, appears to have no anti-apoptotic characteristics, according to our previous observations of unchanged numbers of all cells (mature and precursor) in these two major hemopoietic cells lineages during either 7 or 14 days of dietary administration (Sun et al., 1999).

Inhibition of maturation in these two vital cell lineages (erythroid and granulocytic) is clearly undesirable, given that the mature cells in each lineage are the functional ones. A halt in development

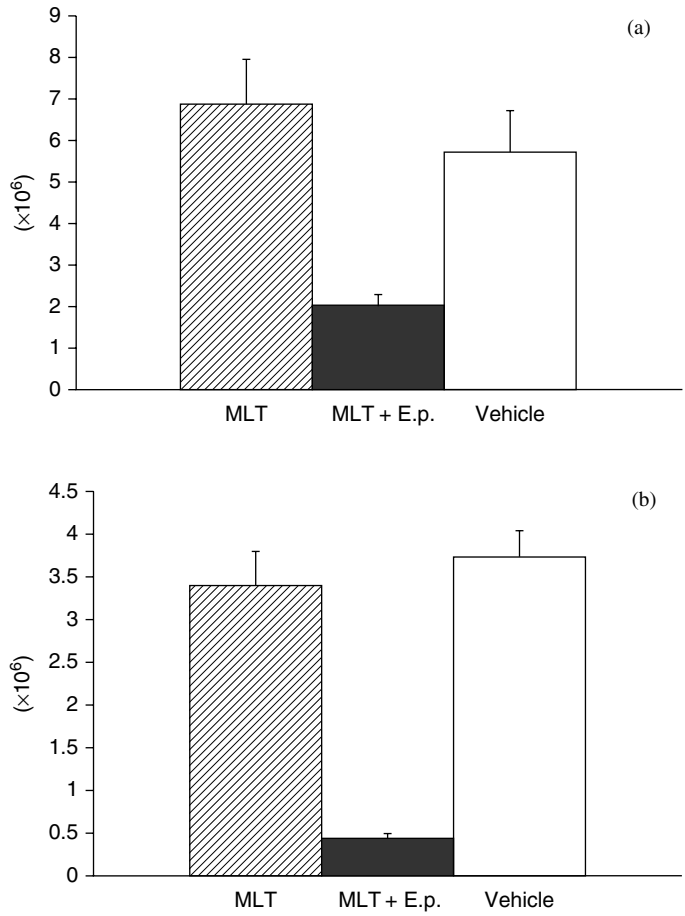

FIGURE 16.2 Absolute numbers of mature, functional granulocytes in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.002$ (MLT vs. MLT + E.p.: spleen); $p < 0.01$ (control vs. MLT + E.p.: spleen); $p < 0.0003$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.004$ (control vs. MLT + E.p.: bone marrow).

and maturation toward such mature, functional cells would obviously compromise the well-being of the animal/human. Anemia would result from a halt in production of mature cells in the erythroid lineage, and disruptions in disease defense mechanisms mediated by granulocytes would ensue in the absence of mature functional granulocytes.

The lymphoid cells of the spleen, which are comprised predominantly of re-circulating, mature, functional antigen-reactive T and B cells, identified as small lymphocytes (< 8.0μ in nuclear diameter), were significantly increased in number when MLT alone was administered in the diet for 14 days (Figure 16.4a), in keeping with the immunoenhancing properties of this neurohormone. Interestingly, however, adding *E. purpurea* along with MLT resulted in a cancellation of this MLT-mediated, significant enhancement of lymphocyte numbers in the spleen (Figure 16.4a), returning them to control (vehicle) levels. In the bone marrow, adding *E. purpurea* along with MLT resulted in a much greater significant reduction in the absolute numbers of these lymphocytes relative to that of mice fed the MLT only diet, or the control diet (Figure 16.4b). The bone marrow is the production site of B lymphocytes, a major population of immune cells, and as such, contains the

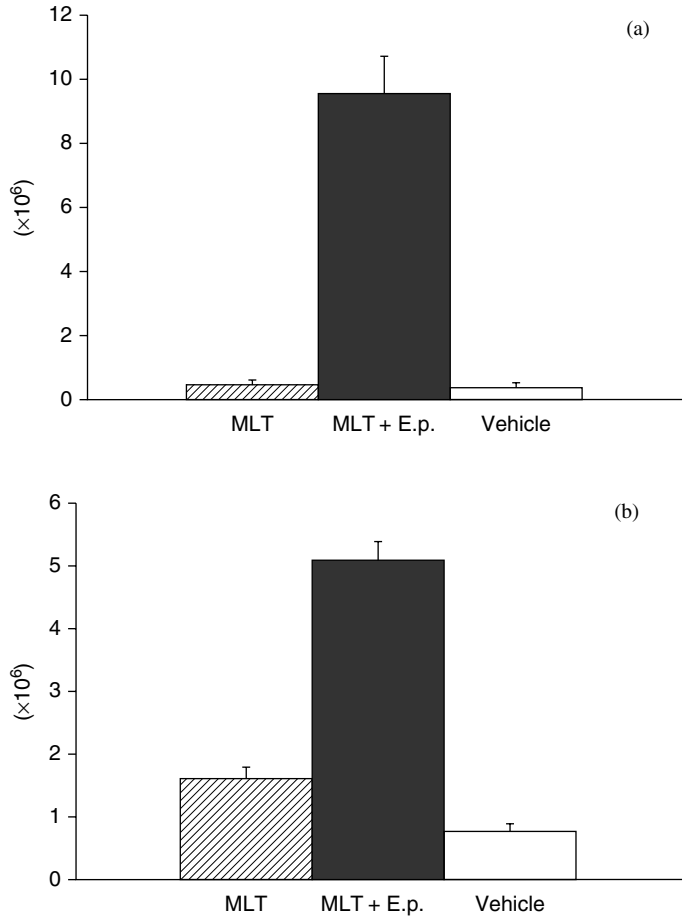

FIGURE 16.3 Absolute numbers of precursor (proliferating) granulocytic cells in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.0003$ (MLT vs. MLT + E.p.: spleen); $p < 0.0003$ (control vs. MLT + E.p.: spleen); $p < 0.02$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.00003$ (control vs. MLT + E.p.: bone marrow); $p < 0.00003$ (MLT vs. control: bone marrow).

proliferating precursors of this lineage. Although these proliferating precursors in the lymphocyte lineage were not separately enumerated in the bone marrow where they exist normally in very low numbers, some disturbance in the production of their mature progeny (small, functional lymphocytes), apparently had occurred in the combined presence of both MLT + *E. purpurea*, given that the observed numbers of small lymphocytes in that organ were very low relative to the control diet or MLT only groups (Figure 16.4b). Thus, it appears that ingestion of both MLT and *E. purpurea* inhibits the production/maturation process of lymphocyte precursors toward their mature functional progeny (small lymphocytes), a phenomenon that parallels that observed with the erythroid and granulocytic lineages (Figure 16.1 through Figure 16.3).

Two crucial cell lineages in the disease defense process, monocyte/macrophage and NK cells, need yet to be considered. Both cell lineages are normally present in very low numbers *in vivo*, and in mice, the absolute numbers of monocyte/macrophage-type cells typically average 1% of all spleen cells (Miller and Kearney, 1997; Sun et al., 1999). Fourteen days of co-administration of MLT + *E. purpurea* significantly reduced the numbers of monocyte/macrophage cells relative to the numbers of such cells found in the spleens of control diet mice (Figure 16.5a). Moreover,

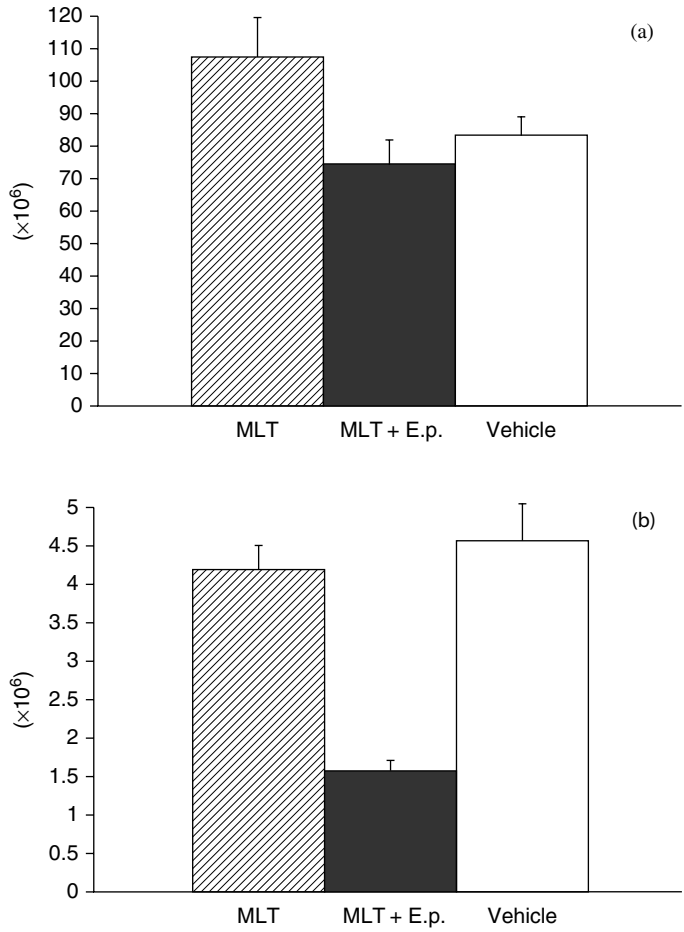

FIGURE 16.4 Absolute numbers of lymphocytes in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.03$ (MLT vs. control: spleen); $p < 0.03$ (MLT vs. MLT + E.p.: spleen); $p < 0.00003$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.0002$ (control vs. MLT + E.p.: bone marrow).

relative to MLT-consuming mice, the numbers of these cells in the spleens of mice consuming both MLT + *E. purpurea* were also significantly lower (Figure 16.5a). In the bone marrow, in parallel, the numbers of monocyte/macrophage cells also fell precipitously in the presence of MLT + *E. purpurea* compared to control diet mice (Figure 16.5b). Relative to MLT only mice, the decrease in monocyte/macrophage numbers in the bone marrow of mice fed both MLT + *E. purpurea* was also very significant (Figure 16.5b). Thus, in parallel with the other lineages (above), it appears that inhibition of production/maturation of mature cells in the monocyte/macrophage lineage may be responsible for the severe subnormal numbers of these cells found in mice that consumed the MLT + *E. purpurea* diet. One additional matter of note is that in the bone marrow, MLT alone significantly increased the number of monocytes/macrophages. The same interpretation here exists as with the proliferating precursor cells in the granulocytic lineage in the bone marrow (Figure 16.3b). That is, MLT stimulates bone marrow stromal cells to produce the hormone GM-CFU. While "G" stands for granulocytes, "M" stands for monocyte/macrophages. Hence, it is not unexpected that cells of the latter lineage are considerably increased in number in the bone marrow in the presence of MLT alone (Figure 16.5b).

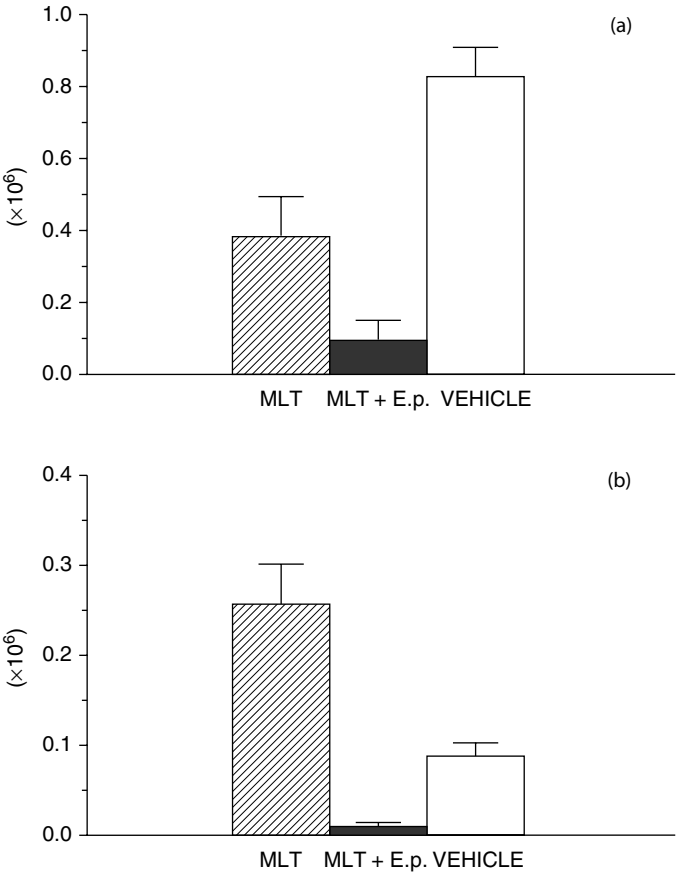

FIGURE 16.5 Absolute numbers of monocytes/macrophages in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.007$ (MLT vs. control: spleen); $p < 0.04$ (MLT vs. MLT + E.p.: spleen); $p < 0.006$ (MLT + E.p. vs. control: spleen); $p < 0.003$ (MLT vs. control: bone marrow); $p < 0.001$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.002$ (MLT + E.p. vs. control: bone marrow).

NK cells, after 14 days of *in vivo* exposure to the combination of MLT + *E. purpurea*, were negatively affected in both the spleen and bone marrow (Figure 16.6a and Figure 16.6b). In the spleen (Figure 16.6a), NK cell numbers were profoundly reduced relative to those of the MLT alone and control diet mice. In the bone marrow (Figure 16.6b), NK cells were equally profoundly reduced by co-administration of the two agents. *E. purpurea* by itself is a powerful stimulator of NK cells; the herb's powerful NK-enhancing properties derive from a mixture of phytochemicals ranging from interferon inducers (Carr et al., 1998; Leuttig et al., 1989) to prostaglandin inhibitors (Wagner et al., 1995). Among others (Currier and Miller, 1998; Dussault and Miller, 1993; Kendall and Targan, 1980; Lala et al., 1986; Minato et al., 1980; Wagner et al., 1995), we have shown that interferon inducers, interferon itself, and any prostaglandin inhibitor all profoundly enhance NK cell numbers and function. Equally powerful as an NK cell stimulant is MLT (Currier et al., 2000). In contrast, however, MLT and *E. purpurea* together appear to have a devastating effect on this fundamental lineage in the disease defense processes, i.e., one which acts as a first line of defense in the face of virus infections and tumors.

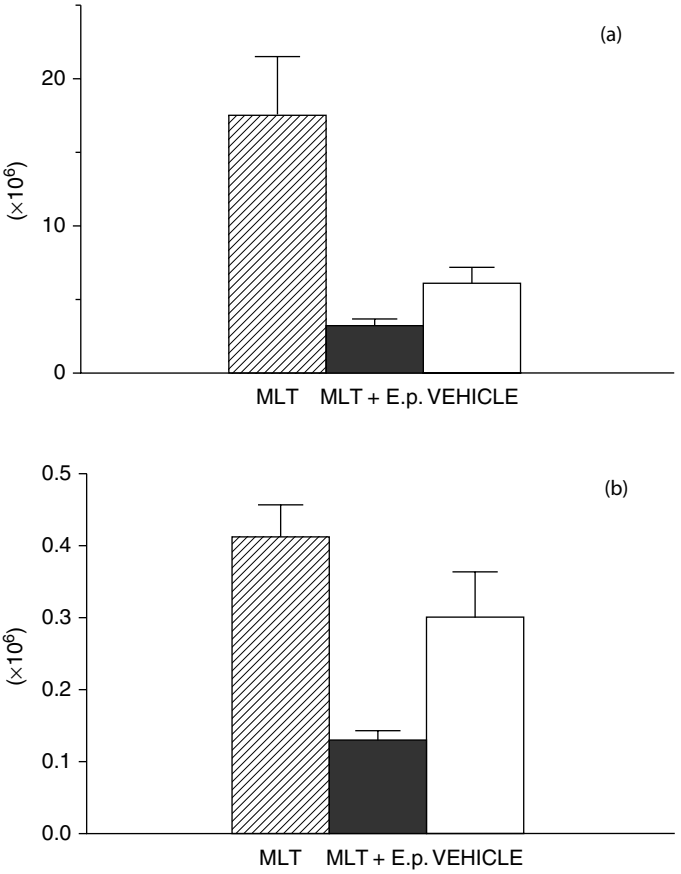

FIGURE 16.6 Absolute numbers of natural killer cells in the spleen (a) and bone marrow (b) of untreated (control) mice, mice given melatonin (MLT) alone, or mice simultaneously administered MLT + *E. purpurea* (E.p.), daily for 14 days. Mean ± standard error: 9 to 10 mice/group. $p < 0.02$ (MLT vs. control: spleen); $p < 0.01$ (MLT vs. MLT + E.p.: spleen); $p < 0.05$ (MLT + E.p. vs. control: spleen); $p = 0.05$ (MLT vs. control: bone marrow); $p < 0.0006$ (MLT vs. MLT + E.p.: bone marrow); $p < 0.04$ (MLT + E.p. vs. control: bone marrow).

Table 16.1 indicates that when MLT + *E. purpurea* were given via diet to mice for only 7 days, the changes in each of the cell lineages under study closely followed those obtained from mice consuming the same agents for 14 days (Figure 16.1 through Figure 16.5). That is, there appears to be a relatively rapid onset (7 days) of the negative influences of simultaneous ingestion.

In summary, it appears from this study that combinations of individually beneficial agents, when taken together, can have profound, far-reaching, and unwanted side effects. That is, natural agents, each of which may have a substantial beneficial effect on health and well-being, should be considered with caution in combination. The present analysis has indicated that at least two health-mediating agents, melatonin and *E. purpurea*, do indeed have detrimental effects when combined. Taken together, these two agents, and possibly others, appear to severely compromise the normal status of the vital hemopoietic and immune systems.

TABLE 16.1
The Effect of 7 Days of Simultaneous Administration of Dietary Melatonin[a] + *E. Purpurea*[b] on Hemopoietic Cell Lineages of Spleen and Bone Marrow

Organ	Nucleated Erythroids ($\times 10^6$)[c]	Mature Granulocytes ($\times 10^6$)	Precursor Granulocytes ($\times 10^6$)	Lymphocytes ($\times 10^6$)	Monocytes/Macrophages ($\times 10^6$)
Spleen	7.11±0.64[d]	2.10±0.31	10.97±0.96	92.38±5.74	0.17±0.06
Bone marrow	2.98±0.30	0.57±0.08	4.88±0.47	1.34±0.08	0.001±0.0

[a] Melatonin given in the ground chow as 14.2 µg/mouse/day.

[b] *E. purpurea* given in the ground chow as 0.45 mg/mouse/day.

[c] Determined from differential counts of 2,000 total nucleated cells/organ (enumerated by means of an electronic cell counter) and converted to absolute numbers of cells in each morphologically identifiable lineage.

[d] Mean ± standard error: 9 to 10 mice.

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17 Adverse Reactions Associated with *Echinacea* and Other Asteraceae

Raymond J. Mullins and Robert Heddle

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INTRODUCTION

Fifty percent of Australians report using some form of complementary alternative medicines (CAM) apart from vitamins in any 12-month period, with similar patterns of use in British and North American subjects (Eisenberg et al., 1993; Maclennan et al., 1996, 2002; Schafer et al., 2002). Despite the common perception that "natural therapy" is safe, toxic and hypersensitivity reactions to CAM have been described (Drew and Myers, 1997; Mullins and Heddle, 2002; Shaw et al., 1997; Vickers and Zollman, 1999). Given that these products are rarely packaged in childproof containers, accidental exposure also occurs (Anderson, 1996; Portansky, 1998). Allergic reactions are most common in atopic subjects. This is not surprising when one considers that up to 20% of atopic subjects use CAM. Furthermore, these patients are more likely than others to become sensitized to cross-reactive allergens and some use (or are advised to use) products such as *Echinacea* for treatment of allergic disease (Healy et al., 2002; Mullins, 1998).

When interpreting reports of immediate hypersensitivity to Asteraceae-derived CAM, it is helpful to bear in mind a number of important concepts: (1) exposure to Asteraceae is common; (2) sensitization is more common in subjects with preexistent allergic disease; (3) there is allergenic cross-reactivity between different Asteraceae, and between Asteraceae and some foods; and (4) patients sensitized by inhalation may experience allergic reactions when exposed by other routes. The implication is that unexpected adverse reactions may occur even with first ever known exposure.

Not all adverse reactions to Asteraceae, however, are IgE mediated. Some patients will experience delayed hypersensitivity. Others experience adverse effects where the mechanism is poorly defined. An important implication is that not all adverse reactions will be confined to atopics, but may extend to others with undefined risk factors. Adverse reactions are summarized in Table 17.1.

IMMEDIATE HYPERSENSITIVITY REACTIONS

With over 20,000 species of Asteraceae distributed worldwide (absent only from the Antarctic mainland (Jeffery, 1978), exposure to inhaled or ingested members of this family is inevitable. *Echinacea* (or coneflower) is a flowering member of the Asteraceae (Compositae) family whose other members include *Ambrosia* (ragweed) species, *Artemisia* (mugwort, sagebrush, wormwood) species, *Parthenium* (feverfew), and cultivated plants including chrysanthemums, dahlias, sunflowers, marigolds, safflower, and daisies (Platts-Mills and Solomon, 1993). Edible plants such as lettuce, safflower, chicory, and artichoke are also Asteraceae. Some members are used as CAM, including *Echinacea*, dandelion, chamomile, feverfew, milk thistle, and wormwood (Newall et al., 1996).

Sensitization to Asteraceae is common. Asteraceae-derived pollens are an important trigger for allergic rhinitis and asthma, including *Ambrosia* (ragweed) in North America, *Parthenium* (feverfew) in South America and India, *Artemisia* (mugwort) in Spain, and *Chrysanthemum* and sunflower in occupational and population settings (Atis et al., 2002; Bousquet et al., 1985; Goldberg et al., 1998; Groenewoud et al., 2002; Jimenez et al., 1994; Kuroume et al., 1975; Negrini and Arobba, 1992; Park et al., 1989; Sriramarao et al., 1991; Uter et al., 2001).

Cross-reactivity between inhaled and ingested allergen is a risk factor for allergic reactions with exposure via other routes (reviewed in Baldo, 1996; Caballero and Martin-Esteban, 1998). Precedents include oral allergy syndrome in pollen-sensitive subjects and some allergic reactions to sunflower seeds and crustaceans (Axelsson et al., 1994; Caballero et al., 1994; Leung et al., 1996). Sensitization to Asteraceae has also been associated with immediate hypersensitivity to CAM, such as royal jelly, *Echinacea*, bee pollen extracts, and chamomile, and some foods such as celery, honey, sunflower seeds, carrot, lettuce, watermelon, and nuts (Angiola Crivellaro et al., 2000; Axelsson et al., 1994; Bauer et al., 1996; Bousquet et al., 1984; Cohen et al., 1979; Dawe et al., 1996; Dietschi et al., 1987; Florido-Lopez et al., 1995; Garcia Ortiz et al., 1996; Helbling et al., 1992; Leung et al., 1995; Lombardi et al., 1998; Reider et al., 2000; Subiza et al., 1989; Vallier et al., 1988; Vila et al., 1998). An appreciation of the concept of cross-sensitization makes unexpected reactions to CAM with first known exposure (such as to chamomile, *Echinacea*, royal jelly and pollen-derived products) perhaps not so surprising after all (Lombardi et al., 1998, Mullins and Heddle, 2002; Subiza et al., 1989).

These observations are consistent with the hypersensitivity reactions to *Echinacea* in Australian subjects (Mullins and Heddle, 2002). Of 26 subjects with immediate hypersensitivity, 4 had anaphylaxis, 12 suffered acute asthma attacks, and 10 experienced urticaria/angioedema. Reactions were not always mild: four were hospitalized, four reacted after their first ever known exposure, and one patient suffered multiple progressive systemic allergic reactions. *Echinacea* was the sole implicated medication in 15 cases.

Consistent with atopy being an important risk factor, over half were known to be atopic. Furthermore, when 100 consecutive atopic patients were skin tested, 20 had positive reactions

TABLE 17.1
Major Adverse Reactions Associated with Asteraceae-Derived Complementary Alternative Medicines

Asteraceae	Immediate Hypersensitvity	Contact Allergic Dermatitis	Other Reactions	Toxicity	Pregnancy	Disease Interactions	Drug Interactions
Calendula				b	Abortificant (e)		
Chamomile	b	b			Teratogenic (c)		Warfarin (d)
					Abortificant (e)		CYP34A (d)
Dandelion	b	b					Diuretics (e)
							Hypoglycaemics (e)
Echinacea	b	b	Hepatitis (b)		Safety (a)	Autoimmune disease	CYP34A (d)
			Rashes (b)			(cde)	Hepatotoxins (e)
			Erythema nodosum (b)			Chronic infection (cde)	
			Delayed asthma (b)			Surgery (cde)	
						Transplantation (cde)	
Feverfew	b	b			Abortificant (bce)		NSAIDS, (d)
							Warfarin (d)
Milk thistle			GIT symptoms and vascular collapse (b)		Abortificant (e)		CYP34A (d)
Royal jelly[a]	bd						
Tansy	b				Abortificant (e)		
Wild lettuce	b			c			
Wormwood	b				Abortificant (e)		
Yarrow					Abortificant (e)		

Note: Summary of levels of evidence for major described adverse reactions, classified according to those provided by (a) case-controlled series; (b) clinical case reports or series; (c) animal studies; (d) *in vitro* studies; and (e) opinions by experts in the field. References are cited in the text.

[a] Royal jelly may contain Asteraceae pollen allergens.

to *Echinacea*, yet only three had ever taken it previously. While this cohort had large positive reactions to grass pollens on skin testing, reactions to *Ambrosia* sp. were either negative or no greater than 2 mm. Given that exposure to ragweed, feverfew, or mugwort pollen in Australia is either sparse or nonexistent, this was not a surprising result. The implication was that sensitization to *Echinacea* must have developed indirectly, by exposure to flowering ornamental Asteraceae, cross-reactive foods, or plants growing in the wild associated with Australian bush dermatitis (see below).

CONTACT ALLERGIC DERMATITIS

Asteraceae are a common cause of occupational contact allergic dermatitis (CAD). *Echinacea*, daisies, chrysanthemum, chamomile, tansy, dandelion, feverfew, and sunflowers have all been associated with symptoms in domestic and market gardeners and florists (de Jong et al., 1998; Goldberg et al., 1998; Ingber, 2000; Mitchell and Rook, 1979; Paulsen, 1998; Paulsen et al., 1997; Pereira et al., 1997; Rodriguez-Serna et al., 1998). Some topical CAM, cosmetics, shampoos, and massage oils containing plant extracts cause similar symptoms (Table 17.1; Bossuyt and Dooms-Goossens, 1994; Gordon, 1999; McGeorge and Steele, 1991).

Contact with airborne plant-derived oleoresins can also cause dermatitis, a condition commonly known as Australian bush dermatitis, ragweed dermatitis, and weed dermatitis. Asteraceae are also responsible for some cases of persistent light eruption (Burke et al., 1996; Dawe et al., 1996). The face, eyelids, sides of the neck, and "V" area of the neck are the main areas affected, with sharp delineation between unaffected areas protected by clothing. Cross-reactive, oil-soluble sesquiterpene lactones are the dominant (but not only) allergens responsible (Ducombs et al., 1990; Goulden and Wilkinson, 1998; Kanerva et al., 2001; Paulsen et al., 2001; von der Werth et al., 1999). Ingestion of lettuce has been associated with aggravation of dermatitis in one report (Oliwiecki et al., 1991). Affected patients are often advised to avoid contact with all Asteraceae (Abt and Hammerly, 2002; Newall et al., 1996).

OTHER ADVERSE REACTIONS

Delayed Asthmatic Reactions

We have personally assessed four patients (one previously reported in Mullins and Heddle, 2002) who developed delayed asthmatic responses following ingestion of *Echinacea*, reproducible on rechallenge in all cases. Whether these observations were due to coincidence, related to why *Echinacea* was taken (e.g., infection), or due to non–IgE-mediated pro-inflammatory properties of *Echinacea* is uncertain (Table 17.1).

Rashes

Twelve cases of nonurticarial rashes (out of 51 reports involving *Echinacea*) were noted in Australian adverse drug reports, and at least one was reproducible with rechallenge (Mullins and Heddle, 2002). A single case report has implicated *Echinacea* as a cause of recurrent erythema nodosum (Table 17.1) (Soon and Crawford, 2001).

Hepatitis

Echinacea contains potentially hepatotoxic pyrrolizidine alkaloids. Hepatitis has been described in 7 of 51 Australian and in U.S. adverse drug reports involving *Echinacea* (Table 17.1) (Mullins and Heddle, 2002; Food and Drug Administration, 1998).

Toxicity

Tansy oil contains ketone beta-thujone, a toxic compound associated with gastritis, seizures, cardiovascular side effects, and death (Tisserland and Balacs, 1995). Its sale is banned in many countries. Overdoses with wild lettuce (sometimes used as a sedative) have been blamed for respiratory depression, coma, and death in cattle (Mabey, 1988).

Other Reported Adverse Reactions

Transient burning or stinging of the tongue is commonly reported after taking *Echinacea* (Blumenthal et al., 1998). Parenteral administration can cause shivering, fever, and muscle weakness (Parnham, 1996). Additional complaints including nausea and constipation (Grimm and Muller, 1999), and abdominal pain, diarrhea, dysphagia, and skin rashes in German adverse drug reports 1989 through 1995 (Parnham, 1996). In the Australian Adverse Drug Reactions Advisory Committee (ADRAC) database, additional symptoms included fatigue, arthralgia, or myalgia (four cases each), headache or hypertension (two cases each), and one case each of dizziness, atrial fibrillation, vasculitis, acute renal failure, nausea, and epistaxis (Mullins and Heddle, 2002).

Mouth irritation, mouth ulcers, reduced taste, dry tongue, and gastric irritation have been reported in those using feverfew (Ernst and Pittler, 2000; Johnson et al., 1985). Milk thistle has been associated with reproducible symptoms of sweating, colicky abdominal pain, diarrhea, vomiting, weakness, and vascular collapse requiring hospitalization in one case report (ADRAC, 1999).

USE IN PREGNANCY, BREAST FEEDING, AND CHILDREN

Given the paucity of published studies, the potential risks and benefits of using CAM during pregnancy or lactation are difficult to assess (Ernst, 2002a). Nevertheless, up to 12% and 55%, respectively, of pregnant Nigerian and South African women have taken native herbs during pregnancy (Gharoro and Igbafe, 2000; Mabina et al., 1997). CAM are also used by 12% to 15% of pregnant American women to relieve morning sickness or treat intercurrent illness, most commonly ginger, chamomile, *Echinacea*, or vitamins (Pastore, 2000; Tsui et al., 2001), a practice associated with congenital lead poisoning in a recent report (Tait et al., 2002). Around half of American midwives in one study used herbal products to induce labor (McFarlin et al., 1999). This has been associated with anaphylaxis and fetal death following administration of a chamomile enema (Jensen-Jarolim et al., 1998). Up to 7% may use CAM during lactation (Hepner et al., 2002).

Despite a Commission E monograph statement that *Echinacea* is safe in pregnancy (Blumenthal et al., 1998), this has only been formally examined in one underpowered study of 206 Canadian women, 54% of whom took *Echinacea* during the first trimester. While no significant increase in the type or incidence of malformations or pregnancy-related complications was found compared to case-matched controls (Gallo et al., 2000), this small study had only the power to detect a major teratogen. The amount of alcohol present in some *Echinacea* preparations [(estimated at around 1 ml per day) (Gallo et al., 2000)] has not been associated with fetal malformations.

There are no published studies examining the safety of other Asteraceae-derived products during pregnancy, yet the properties of some suggest they should be avoided. For example, feverfew is documented to trigger abortions in cattle and stimulate uterine contractions in pregnant women (Farnsworth, 1975). Chamomile is teratogenic in animal studies (Habersang et al., 1979). Safflower, tansy, feverfew, calendula, chamomile, yarrow, milk thistle, and wormwood promote menstruation, stimulate uterine contraction, and act as an abortificant (Ernst, 2002a; Newall et al., 1996). Taken together with the potential for allergic reactions in susceptible individuals, the use of Asteraceae-containing CAM during pregnancy seems imprudent. Similarly, there are few studies of their use in infants and children (Abt and Hammerly, 2002; Newall et al., 1996). Despite this, a recent South Australian survey showed that 87% of children admitted to Adelaide's Women's and Children's

Hospital had received at least one CAM in the previous 12 months, and that 16% had received six or more preparations (Maclennan et al., 2002). Given the unsupervised use of CAM by many patients and tendency to underreport adverse reactions to medication of all types, the absence of published evidence of toxicity in this or other groups should not be interpreted as evidence of safety (Myers, 2002).

DRUG INTERACTIONS, CONTRAINDICATIONS, AND PRECAUTIONS

Patient survey data from Canada, the U.S., and Australia show that one in five patients use prescription drugs concurrently with CAM. The inherent polypharmaceutical nature of CAM increases the risk of adverse events if these CAM either have pharmacological activity or interfere with drug metabolism (Ackerman et al., 1999; Bensoussan and Myers, 1996; Smolinske, 1999). Since confirmed interactions are sporadic and based largely on case reports, advice to avoid certain drug–CAM combinations is based on known pharmacological and *in vitro* properties (Klepser and Klepser, 1999; Miller, 1998; Myers, 2002; Scott and Elmer, 2002; Shalansky, 2001). Major adverse reactions associated with Asteraceae-derived CAM are summarized in Table 17.1, with levels of evidence for these classified according to those provided by (1) case-controlled series, (2) clinical case reports or series, (3) animal studies, (4) *in vitro* studies, and (5) opinions by experts in the field.

Known Hypersensitivity to Asteraceae

Cross-reactive sesquiterpene lactones are present in many, if not all, Asteraceae. Patients with known CAD from one plant may develop similar type IV reactions following contact with others (Dawe et al., 1996; Goldberg et al., 1998; Gordon, 1999; Mitchell and Rook, 1979). Affected patients are often advised to avoid contact with all Asteraceae (Abt and Hammerly, 2002; Newall et al., 1996), yet this advice is based on limited knowledge of cross-reactivity between relatively few members of this large family.

Some authorities recommend avoiding Asteraceae-derived CAM if, for example, the patient is known to have IgE-mediated inhalant allergy to ragweed (Abt and Hammerly, 2002; Newall et al., 1996). While a reasonable approach, this ignores a number of important facts: (1) many atopic patients are unaware of their sensitization "profile"; (2) the degree of cross-reactivity among over 20,000 species of Asteraceae, and between Asteraceae and superficially unrelated plants, remains undefined; (3) even patients without defined sensitization to inhaled Asteraceae may react to CAM like *Echinacea*; and (4) atopics are more likely than other patients to suffer allergic reactions to CAM, even with first known exposure. It thus seems more prudent to advise caution in all (not just a subset) of atopic subjects.

Liver Disease

The presence of pyrrolizidine alkaloids in *Echinacea* together with reports of hepatitis have led to advice to avoid potentially hepatotoxic medication in combination with *Echinacea*, such as anabolic steroids, methotrexate, ketoconazole, or amiodarone (Miller, 1998). Perhaps more important is the potential for all of these substances to influence drug metabolism by inhibiting hepaticCYP3A4 activity.

Interference with Hepatic CYP3A4 Enzyme Activity

CYP3A4 is one of approximately 50 individual cytochrome P450 enzymes playing an important role in drug metabolism, an activity inhibited by *Echinacea*, milk thistle, and chamomile (Budzinski et al., 2000; McKinnon and Evans, 2000). In theory, these products could interfere with metabolism

TABLE 17.2
Medications Interacting with CYP3A4

Enzyme Substrates	Enzyme Inhibitors
Alprazolam, amiodarone, amitriptyline, astermizole, atorvastatin, budesonide, buprenorphine, busulphan, carbamazepine, cisapride, clarithromycin, clomipramine, clonazepam, clozapine, cocaine, cortisol, cyclophosphamide, cyclosporin, dapsone, dexamethasone, dextromethorphan, digitoxin, diltiazem, diazepam, doxorubicin, erythromycin, ethinyloestradiol, ethosuximide, etoposide, felodipine, fentanyl, fexofenadine, flutamide, ifosfamide, imipramine, indinavir, ketoconazole, loratadine, losartan, lovastatin, miconazole, midazolam, nifedipine, nelfinavir, oestradiol, omeprazole, ondansetron, paclitaxel, propafenone, quinidine, ritonavir, saquinavir, sertraline, simvastatin, tacrolimus, tamoxifen, teniposide, tetrahydrocannabinol, theophylline, trazadone, troleandomycin, verapamil, vinblastine, vincristine, warfarin	Amiodarone, cannabinoids, cimetidine, clarithromycin, clotrimazole, delavirdine, diltiazem, erythromycin, fluoxetine (due to norfluoxetine metabolite), fluvoxamine, grapefruit juice, itraconazole, ketoconazole, metronidazole, miconazole, nefazodone, paroxetine, protease inhibitors, troleandomycin

of medications such as calcium channel blockers, antihistamines, or antiretroviral agents as summarized in Table 17.2 (Flockhart, 2001; McKinnon and Evans, 2000). Potential interactions are even more important when using medications like amiodarone, cisapride, carbamazepine, cyclosporine, warfarin, or antiretroviral agents, all of which have narrow therapeutic windows.

Anticoagulant Use

Feverfew inhibits cyclooxygenase and phospholipase A2, potentially increasing the risk of bleeding while taking oral anticoagulants or aspirin (Makheja and Bailey, 1982; Sumner et al., 1992). Chamomile contains coumarins that may potentiate warfarin activity (Heck et al., 2000; Hoult and Paya, 1996; Miller, 1998; Myers, 2002).

Autoimmune Disease and Chronic Infection

Because of its purported short-term immunostimulatory effect, some authorities recommend that *Echinacea* be avoided in patients with autoimmune disease (e.g., systemic lupus erytematosus, multiple sclerosis), or in those with chronic HIV infection or tuberculosis (Chavez and Chavez, 1998; Ernst, 2002b; Miller, 1998; Newall et al., 1996). Such statements are not evidence based. For example, while *Echinacea*-associated TNF release might conceivably aggravate rheumatoid arthritis, it could actually benefit patients with active tuberculosis, where TNF appears to exert a protective role (Kalden, 2002; Smith et al., 2002).

Tissue Transplantation and Surgery

Because of its purported immunostimulatory effect, it is commonly recommended that *Echinacea* be avoided in patients undergoing organ transplantation, to reduce the risk of rejection. Similarly, *Echinacea* is thought to inhibit wound healing and thus might interfere with surgical recovery (Chavez and Chavez, 1998; Miller, 1998; Ang-Lee et al., 2001). Medications with a potential anticoagulant effect such as feverfew or chamomile should probably also be avoided (Makheja and Bailey, 1982; Myers, 2002; Sumner et al., 1992).

Interaction with Alcohol, Diuretics, and Hypoglycemic Agents

Combining alcohol-containing tinctures of *Echinacea* with disulfuram or metronidazole is not advised by some authorities (National Standard, 2002), although the amounts of alcohol ingested are such that the risk is remote. Artichoke and dandelion are purported to have diuretic activity (Miller, 1998) and the latter may have some hypoglycaemic activity as well (Abt and Hammerly, 2002).

CONCLUSIONS

The increasing popularity of CAM and concurrent use of conventional medication makes it increasingly likely that otherwise rare adverse reactions or drug interactions will occur. Administration of CAM is largely unsupervised. Doctors and patients may find it difficult to distinguish symptoms due to disease from those secondary to treatment. Many medical practitioners are ignorant of the potential toxicity of CAM. These factors, together with underreporting of use by patients (Maclennan et al., 2002), may contribute to underreporting of adverse events.

The safety of any product is a relative concept that takes into account the potential for toxicity in the entire population as a whole, as well as those at particular risk by virtue of age, sex, organ dysfunction, or atopy. Atopic patients appear to be at particular risk of allergic reactions of variable severity to Asteraceae-derived CAM, even with first ever known exposure. Patients should be warned appropriately. Consideration should be given to attaching warning labels similar to those currently attached to aspirin and royal jelly packets in some countries.

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18 The Efficacy of *Echinacea* Tea

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INTRODUCTION

Herbal remedies continue to grow in popularity in the U.S. as demonstrated by expanding sales with seemingly no correlation to scientific research. *Echinacea* preparations have developed into the best-selling herbal immunostimulants (Bauer, 1998). Nine species of the genus *Echinacea* are found today in the U.S. and Canada (McGregor, 1968). Native Americans used *Echinacea* to treat wounds, snakebites and other animal bites, tonsillitis, headache, and cold symptoms (Hobbs, 1989). In the early 1900s in the U.S., *Echinacea* was the most utilized indigenous medicinal plant. After the introduction of antibiotics, its use declined in the U.S., although today it remains popular in Europe (Foster, 1991).

Although *Echinacea* is processed and sold around the world, Switzerland and Germany have been in the forefront by marketing more than 800 homeopathic products and drugs containing *Echinacea* (Brevoort, 1996). Analyses of these preparations have shown that three different species of *Echinacea* are used in medicine and homeopathy: *Echinacea angustifolia* DC, *Echinacea pallida* (Nutt.) Nutt., and *Echinacea purpurea* (L.) Moench. (Asteraceae) (Bauer, 1998). Even though a number of species of *Echinacea* have shown an immunostimulating effect, *E. purpurea* has been the type most often used for relief of symptoms of flu, cold, and upper respiratory illnesses (Melchart et al., 1995; Burger et al., 1997). When the aqueous extracts of the aerial parts of the *E. purpurea* were subjected to systematic fractionation and pharmacological testing, the result was the isolation of two polysaccharides with immunostimulating properties (Wagner and Proksch, 1981). These polysaccharides were found both to stimulate phagocystosis *in vitro* and *in vivo*, and to augment the production of oxygen radicals by macrophages in a dose-dependent manner (Stimpel et al., 1984). Problems with analyses of these components continue since the methods are still evolving (Bauer, 1998). Polysaccharides in *Echinacea* are analyzed through specific determination by isolation and structure elucidation or by nonspecific determination by hydrolysis of monosaccharides; neither of these methods is yet commercially obtainable (Bauer, 1998). *E. angustifolia* (a component of Echinacea Plus®) has also been shown, when combined with other types of *Echinacea*, to have an immunostimulating effect in relieving cold and flu symptoms (Melchart et al., 1995).

Researchers have studied various time intervals for *Echinacea* in the prevention and treatment of cold and flu symptoms. Some differences have been determined in research findings on the efficacy of *Echinacea* as a prophylactic over time. In a 6-month double-blind placebo study, the *Echinacea* treatment group had fewer respiratory reinfections (19% vs. 32%), an increase in time interval between such reinfections (25 vs. 40 days), a reduction in the average length of colds (5.3 vs. 7.5 days), and less severe symptoms (Schoneberger, 1992). Grimm and Müller (1999), however, found that *Echinacea* taken prophylactically during a 3-month period did not significantly decrease

the incidence, duration, or severity of colds compared to a placebo. There is also conjecture, but no formal research findings, that *Echinacea* reduces the strength of the immune system response when used continuously over time (Jurcic et al., 1989).

Recent research has to a great extent concentrated on early *Echinacea* intervention for relieving the symptoms and duration of colds and flu-like infections. For example, Bräunig et al. (1992) demonstrated a statistically significant improvement of symptoms over placebo with early intervention, that is, when symptoms first appear. Hoheisel et al. (1997) in a randomized, double-blind, single-center placebo-controlled study demonstrated that the use of an expressed juice of *E. purpurea,* given orally from the onset of the initial symptoms of an upper respiratory infection or cold, inhibits the full expression of the infection and, moreover, shortens the recovery time. Melchart et al. (1994) conducted a meta-analysis of 26 controlled clinical trials (18 randomized, 11 double blind) on the immunomodulatory effects of *Echinacea*; 16 of the 18 randomized trials claimed positive results, suggesting that preparations containing extracts of *Echinacea* can be clinically effective immunomodulators. Melchart et al. (1998) subsequently reviewed 16 trials and found that some *Echinacea* compounds may have stronger effects than a placebo. Most of the research studies showed positive results but without enough evidence to support any specific *Echinacea* product. Another issue has been that of the efficacy of tablet vs. liquid extract. Quite a few articles and research studies have reviewed the digestibility and absorption of the tablet forms of *Echinacea*. The majority of researchers agree that the liquid forms, either in a tea or an alcohol base, are best for maximum absorption and thus maximum efficacy (Wichtl and Bisset, 1994; British Herbal Medicine Association, 1996).

In a research study, Lindenmuth and Lindenmuth (2000) tested the efficacy of an *Echinacea*-compound herbal tea compound on duration and severity of symptoms of cold and flu — specifically, scratchy throat, runny nose, and fever — using a randomized double-blind study. Subjects of the study were employees of Rest Haven–York Nursing and Rehabilitation Center, a 167-bed facility in York, PA. Employees were eligible for the study if they reported the earliest symptoms of cold or flu: runny nose, scratchy throat, or fever. Persons excluded from the study were pregnant women, nursing mothers, those with known allergies to coneflowers, those who stated that they were allergic to any flowering plants or pollens, and those with acute infections and already taking antibiotics.

In December 1998, employees of the nursing and rehabilitation center were advised of the study and received information sheets about *Echinacea*. They were informed that from 1 January 1999 through 30 March 1999, at the earliest sign of a cold or flu symptoms (runny nose, scratchy throat, fever) they could on a voluntary basis be participants in an experimental research study for the purpose of testing the effectiveness of *Echinacea*. Those persons with symptoms who volunteered to be in the study were then randomly assigned throughout the time period to either the experimental group (*Echinacea*) or control group (placebo). The assignment was conducted by specially trained secretarial personnel not associated with the study who had no knowledge of which of two boxes contained packets of Echinacea Plus or Eaters Digest (placebo) tea bags. Upon reporting to the secretarial personnel, each subject received a packet containing 21 tea bags of like appearance (wrappings) of either Echinacea Plus or the placebo. Subjects were assigned numbers, as were the boxes of tea bag packets.

The herbal dietary supplement, Echinacea Plus, was prepared and packaged by Traditional Medicinals®, Inc. of Sebastopol, CA. Echinacea Plus contains a proprietary blend of the leaves, flowers, and stems of organically grown *E. purpurea* and *E. angustifolia*, plus a water soluble dry extract of *E. purpurea* root (6:1). In combination, this delivers the equivalent of 1.275 mg of dried herb and root per tea bag serving. When prepared according to label directions, a minimum of 31.5 mg of total phenolic compounds (calculated as caftaric acid, cichoric acid, chlorogenic acid, and echinacoside) are yielded into one dose of brewed tea, as determined by high-performance liquid chromatography (HPLC). The herbal mixture additionally contains small amounts of two adjuvant components, lemongrass leaf (*Cymbopogon citratus* [DC. ex Nees] Stapf.) and spearmint leaf

(*Mentha spicata* L.). At higher dosages these components might have an effect; however, both lemongrass leaf and spearmint leaf occur in the formula as "flavor corrigents," which are allowed in an herbal tea formula at up to 20% of the formula. This makes the tea palatable in order to ensure patient compliance and tolerance; mint leaf is a widely used flavor corrective in medicinal herbal preparations. (Schilcher, 1997; Weiss, 1988). Specific instructions for boiling, steeping, and dosage were given to each subject as follows: Pour 8 oz. of boiling water over one tea bag and steep, covered, for 10 to 15 minutes. Drink 5 to 6 cups on the first day of symptoms, titrating to 1 cup by the 5th day (Lindenmuth and Lindenmuth, 2000).

The placebo for the control group, "Eater's Digest" herbal tea from Traditional Medicinals, was chosen because the product promotes healthy digestion and has no history of having any effect on cold or flu symptoms. The cinnamon, ginger, and peppermint inclusions could possibly have an effect if given in a higher dosage; in the indicated amounts, they serve as flavor correctives (Schilcher, 1997; Weiss, 1998). Moreover, this tea contains no stimulants and has no obvious or easily recognizable aroma or flavor characteristics that would cause it to be easily discernible from the Echinacea Plus tea by a person with an untrained palate. Subjects would not be likely to have the capability to determine the taste of the *Echinacea* compound, especially since the tea is a multiherb formula containing mint leaf. Furthermore, both the treatment group and control group teas in this study contained mint leaf. A natural flavor could have been added to both teas in an attempt to mask any recognizable or known characteristic flavors, but the problems with that approach are twofold. Subjects may have needed to believe that they were drinking medicinal herbal tea; a flavored tea might have had an influence on the results due to generally not being perceived as medicinal. Second, by adding an ingredient to the existing formula, the drug being studied is no longer the same as the drug in commerce (Echinacea Plus). Additionally, each tea bag (treatment and control) was specially wrapped in the same lining and paper to prevent olfactory and visual differentiation by subjects.

The control tea contained peppermint leaf (*Mentha* × *piperita* Linne); sweet fennel seed (*Foeniculum vulgare* Miller ssp. *vulgare*, var. *dulce* [Miller] Thellung); ginger rhizome (*Zingiber officinale* Roscoe); rose hip (*Rosa canina* L.); papaya leaf (*Carica papaya* L.); alfalfa leaf (*Medicago sativa* L.); and cinnamon bark (*Cinnamomum cassia* J. Presl.) (Lindenmuth and Lindenmuth, 2000). Directions for preparation given subjects in the control and treatment groups were the same.

Bräunig and Knick (1993) reported a study in which a daily dosage of 90 drops of a hydroalcoholic tincture (1:5) (equal to 900 mg of dried *Echinacea* root) is effective in reducing cold-type symptoms in comparison to a daily dosage of 450 mg of dried *Echinacea* root in a second group. Traditional Medicinals recommends 3 to 5 cups per day of the *Echinacea* formula, containing 1000+ mg of *Echinacea* per cup of tea (one bag). The protocol for this study (Lindenmuth and Lindenmuth, 2000) was established at 5 to 6 cups of the tea on the first day of symptoms, titrating to 1 per day for the last of the 5 days. The control (placebo) group was placed on the same schedule.

A questionnaire was designed in a brief format in order to encourage subjects to not only complete it but also to do so with accuracy. It was administered to each subject 14 days after having started the program. Question 1 addressed the effectiveness of the tea in relieving cold or flu symptoms; question 2 requested the number of days that cold and flu symptoms lasted; question 3 asked for the time it took for the subjects to notice any difference in their symptoms. (See Table 18.1 for questionnaire and Table 18.2 for scoring code.) Our hypotheses anticipated a significant difference in effectiveness of relieving cold or flu symptoms between the experimental group (*Echinacea*) and control group (placebo), a significant difference between the experimental group (*Echinacea*) and control group (placebo) in the number of days the symptoms lasted, and a significant difference between the experimental group (*Echinacea*) and control group (placebo) in the number of days it took for subjects to notice a change. Means, standard deviations (SDs), and *t*-tests were run for each question. Confidence intervals of 95% were utilized with statistical significance set at $p < 0.05$ level (two-tailed).

TABLE 18.1
***Echinacea* Study Questionnaire**

1. Please rate on the following scale the effectiveness of the tea in relieving your cold and/or flu symptoms:

1	2	3	4	5
(Not effective)	(Fair)	(Medium)	(Good)	(Excellent)

2. Please circle the number of days your cold and flu symptoms lasted:

Less than 5	6	7	8	More than 10

3. Please circle the number of days it took before you began to notice a difference in your symptoms:

Immediately	2	3	4	More than 5	Not at all

Source: Lindemuth, F. and Lindemuth, E. *J. Alternative Complementary Med.,* 2000. With permission.

TABLE 18.2
***Echinacea* Study Questionnaire: Coding**

Question 1 responses	1 (not effective)	2 (fair)	3 (medium)	4 (good)	5 (excellent)	
Codes	(1)	(2)	(3)	(4)	(5)	
Question 2 responses	Less than 5	6	7	8	More than 10	
Codes	(5)	(4)	(3)	(2)	(1)	
Question 3 responses	Immediately	2	3	4	More than 5	Not at All
Codes	(5)	(4)	(3)	(2)	(1)	(0)

Note: Numbers in parentheses show numerical coding for statistical analysis. The coding was arranged in such a way as to prevent an effect from participants carelessly checking answers on one side.

Source: Lindemuth, F. and Lindemuth, E., *J. Alternative Complementary Med*., 2000. With permission.

The experimental group was comprised of 48 people, and the control group, 47. Ninety-three percent of all employees were women; women accounted for 41 and 40 subjects in the experimental and control groups, respectively. Age ranged from 24 to 62, with a mean age of 39.7, a median age of 40, and a mode of 28. Subjects included RNs, LPNs, maintenance personnel, nurses' aides, dietary staff, therapists, administrators, accountants, and MDs. Rest Haven–York Nursing and Rehabilitation Center is located between an inner city area and a suburban area. The participants live in suburban and rural areas (60%) and the inner city (40%). All subjects who began the study completed the tea regimen and the questionnaire, and were included in the analyses. All subjects reported that they followed the dosage directions exactly (Figure 18.1).

The statistical analyses of the elements of the questionnaire showed the following:

Question 1: There was a significant difference between the experimental group (*Echinacea*) vs. the control group (placebo). Experimental group mean = 4.125, SD = 0.9593; control group mean = 2.787, SD = 0.9541; $t = 6.814$; $p < 0.001$.

Question 2: There was a significant difference between the experimental group (*Echinacea*) vs. the control group (placebo). Experimental group mean = 4.333, SD = 0.9302; control group mean = 2.340, SD = 1.088; $t = 9.499$; $p < 0.001$.

Question 3: There was a significant difference between the experimental group (*Echinacea*) vs. the control group (placebo). Experimental group, mean = 3.854, SD = 0.9735, control group, mean = 2.297, $t = 6.865$; SD = 1.204, t = 6.865; $p < 0.001$ (Lindenmuth and Lindenmuth, 2000).

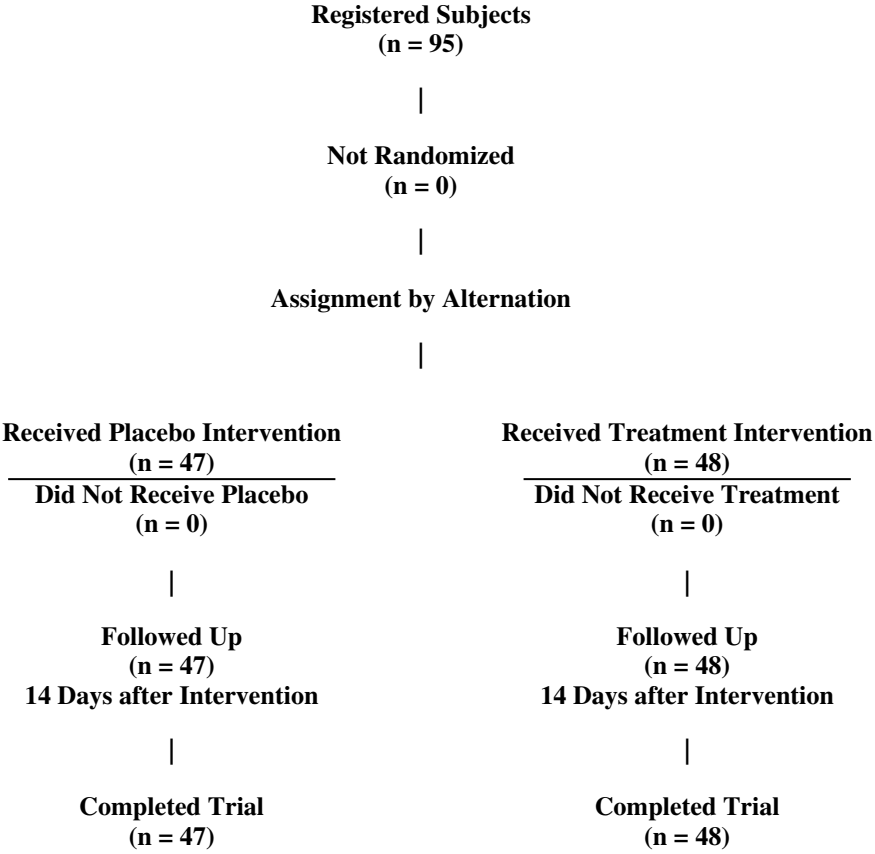

FIGURE 18.1 A study-profile flow chart of participation.

DISCUSSION

The efficacy of *E. purpurea* and *E. angustifolia* combined in tea form was tested in this randomized double-blind placebo-controlled experiment (Lindenmuth and Lindenmuth, 2000). The issues were the evaluation of any changes in the severity of cold or flu symptoms and any change in the amount of time for an effect to appear. Statistically evaluated treatment with the *Echinacea* compound tea taken at the early onset of symptoms was effective at relieving the severity of cold or flu symptoms in a shorter period of time and was noticeably quicker than the placebo in number of days until it had an effect.

During the introduction of the study to the staff, which included the distribution of educational material on *Echinacea*, the intent was that all potential subjects (whether in treatment or control groups) would be under the impression that the tea would have an effect. This along with randomization and scrutinized, effective controls in the double-blind study reduced the likelihood of extraneous errors. Thus, the differences between the two groups found on the three questions are more likely derived from the independent variable than error or chance.

Interviews after the completion of the questionnaire showed that those in the *Echinacea* group reported that their acute symptoms of stuffiness, scratchy throat, and fever seemed to subside within a day or two and with only a "slight drip" remaining. The control group, however, reported acute symptoms lasting 6 to 10 days with little or no relief. None of the subjects reported any side effects. The Rest Haven–York Nursing and Rehabilitation Center experienced 28.7% less absenteeism than in the previous year. These results were not tested for statistical significance due to confounding

variables such as varying severity in different flu and cold seasons and changes in the employee population over the study period.

The findings of the study must be interpreted within the context of the study's limitations. The sample was not representative of the population. Since participation in this study was almost exclusively female, it may be inappropriate to generalize the results in terms of both males and females. There is also the concern of all subjects working in the healthcare field and due to that perhaps differing from the general population. A further limitation may be found in the alternation of the assignment process for group formation; it was accomplished in this manner, first, because the ultimate sample size, i.e., individuals with symptoms, could not be predetermined and second, for the maintenance of the double blind and ease for research assistants conducting the study. The questionnaire has shortcomings due to design simplicity, chosen in order to encourage completion of the survey by all subjects irrespective of their educational level. Although limiting, an assessment device without evaluation of level of symptoms was beneficial for the purpose of not confounding the results with extraneous variables such as degrees of cold or flu. A further limitation is associated with the concept of the self-report device; the self-reporting of cold and flu symptoms and their relief has the usual problems associated with self-report methods. Since self-reports were also used in the control group, this bias is perhaps minimized.

Healthcare professionals and the general public have been seeking more scientific research studies on herbal remedies. It appears at this time that when administered at early onset of symptoms, Echinacea reduces the duration and alleviates cold and flu symptoms. Future empirical studies are needed to continue to evaluate the benefits of Echinacea through manipulation of type of Echinacea, of dosage amount, and of dosage timing. As more research is completed and published there will be increased understanding in terms of efficacy.

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TABLE 19.1
Effect of *Echinacea*

	Mecadox	*Echinacea*%			
		0.0	**0.1**	**0.5**	**2.0**
		Week 0–2			
ADG, kg	0.20	0.16	0.17	0.18	0.17
ADF, kg	0.33	0.31	0.29	0.29	0.29
F/G[ab]	1.62	1.93	1.71	1.62	1.65
		Week 0–3			
ADG, kg	0.25	0.22	0.23	0.24	0.24
ADF, kg	0.41	0.39	0.38	0.38	0.39
F/G[c]	1.66	1.79	1.65	1.57	1.59
		Week 0–4			
ADG, kg	0.32	0.29	0.30	0.30	0.31
ADF, kg	0.51	0.49	0.48	0.48	0.50
F/G[d]	1.60	1.71	1.62	1.58	1.58
		Week 0–5			
ADG, kg	0.38	0.35	0.35	0.36	0.37
ADF, kg	0.64	0.61	0.59	0.60	0.61
F/G	1.65	1.73	1.68	1.65	1.66

[a] Mecadox vs. 0.0%, $p < 0.05$.
[b] $p < 0.05$; vs. 0.5% and 2.0%, $p < 0.01$.
[c] 0.0% vs. 0.5% and 2.0%, $p < 0.05$.
[d] vs. 0.5% and 2.0%, $p < 0.02$.

nursery period when compared to 0% and 1.5% levels, and supported gains equal to the Mecadox diet.

This series of experiments is an important contribution to the database of veterinary applications of *Echinacea*, as it specifically compares *Echinacea* application with that of subtherapeutic antibiotics. Results from these experiments suggest that *Echinacea* may be a good substitute for antibiotics in feed, and results in equal or better performance parameters. It would be of great value to repeat these studies with an *Echinacea* product that is both standardized and characterized, in order to enhance repeatability by other investigators.

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TABLE 19.1
Effect of *Echinacea*

	Mecadox	*Echinacea*%			
		0.0	**0.1**	**0.5**	**2.0**
		Week 0–2			
ADG, kg	0.20	0.16	0.17	0.18	0.17
ADF, kg	0.33	0.31	0.29	0.29	0.29
F/G[ab]	1.62	1.93	1.71	1.62	1.65
		Week 0–3			
ADG, kg	0.25	0.22	0.23	0.24	0.24
ADF, kg	0.41	0.39	0.38	0.38	0.39
F/G[c]	1.66	1.79	1.65	1.57	1.59
		Week 0–4			
ADG, kg	0.32	0.29	0.30	0.30	0.31
ADF, kg	0.51	0.49	0.48	0.48	0.50
F/G[d]	1.60	1.71	1.62	1.58	1.58
		Week 0–5			
ADG, kg	0.38	0.35	0.35	0.36	0.37
ADF, kg	0.64	0.61	0.59	0.60	0.61
F/G	1.65	1.73	1.68	1.65	1.66

[a] Mecadox vs. 0.0%, $p < 0.05$.
[b] $p < 0.05$; vs. 0.5% and 2.0%, $p < 0.01$.
[c] 0.0% vs. 0.5% and 2.0%, $p < 0.05$.
[d] vs. 0.5% and 2.0%, $p < 0.02$.

nursery period when compared to 0% and 1.5% levels, and supported gains equal to the Mecadox diet.

This series of experiments is an important contribution to the database of veterinary applications of *Echinacea*, as it specifically compares *Echinacea* application with that of subtherapeutic antibiotics. Results from these experiments suggest that *Echinacea* may be a good substitute for antibiotics in feed, and results in equal or better performance parameters. It would be of great value to repeat these studies with an *Echinacea* product that is both standardized and characterized, in order to enhance repeatability by other investigators.

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Section VII

The Use of Echinacea *Products in Veterinary Practice*

19 Veterinary Applications of *Echinacea* Species: Research in Horses, Cattle, Poultry, and Swine

Wendy Pearson

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INTRODUCTION

Ethnoveterinary medicine has been defined as "local or indigenous knowledge and methods for caring for, healing, and managing livestock" (Mathius-Mundy and McCorkle, 1989). The concept of using natural therapies in the mitigation of disease and maintenance of health is not new. However, a new landscape of animal husbandry, and in particular the movement away from antibiotics in livestock feed, has created a whole new incentive and urgency to quantifying the usefulness of botanicals in animal diets. As arguably the most popular herbal medicine in the world, *Echinacea* has been widely researched in laboratory animals for its potential clinical uses. However, research in livestock is at best limited. Moreover, as is often the case with botanicals research, access to scientific literature may be inhibited by language of publication. Despite this fact, by virtue of its overwhelming acceptance into human healthcare as an immune system stimulant, *Echinacea* has become a common veterinary contrivance for supporting immune function in livestock. However limited, data do exist which provide species-specific information on the pharmacology, toxicity, and clinical applications of *Echinacea* to various livestock species, including poultry, cattle, horses, and swine.

THE ANTIBIOTICS CONTROVERSY

For decades, it has been commonplace for farmers to incorporate antibiotics into their husbandry strategy. High doses are used to treat recurring infectious diseases that are common to contemporary

livestock housing conditions. However, low maintenance doses are also widely adopted, for the purpose of preventing the spread of infectious disease, and for mimicking the action of growth promoters (Burnell et al., 1988). This strategy has allowed for intensive livestock production without a concomitant decrease in overall herd health. However, it provides for the possible emergence of antibiotic resistant microorganisms in the food chain, which can be transferred to humans (Bates, 1997; van den Bogaard and Stobberingh, 2000). For this reason, in 1998 the European Union banned the use of all antibiotics important in human medicine for use as growth promoters in livestock production. Although this decisive action has not yet been taken in the U.S. or Canada, the possibility holds critical implications for yields and general herd health in many livestock industries, leaving producers seeking alternatives to make up the difference (Bach Knudsen, 2001; Lowenthal et al., 2000).

Echinacea has been extensively investigated in laboratory animals, particularly mice, but also in most of the major classes of livestock, including swine, poultry, cattle, and horses. Practical applications are predominately, although not exclusively, associated with immune system stimulation.

GENERAL PHARMACOLOGY IN LABORATORY ANIMALS

Basic laboratory research into *Echinacea* as a medicinal botanical identifies its major activity as a stimulant to the phagocytic potential of polymorphonuclear cells (Roesler et al., 1991a, 1991b; Steinmüller et al. 1993; Wagner et al., 1988), which is a well-established measurement of immune function (Athlin et al., 1991). Other immunologically relevant activities of *Echinacea* plants include inhibition of cyclooxygenase and 5-lipoxygenase enzymes (Müller-Jakic et al., 1994), two key enzymes involved in the inflammatory response, and a stimulatory effect on the secretion of various cytokines, including IL-1, IL-6, and TNF- (Lüttig et al., 1989; Roesler et al., 1991a, 1991b; Steinmüller et al., 1993). The plant also appears to be an effective antioxidant, and topical mixtures have been shown to provide significant protection against free radical degradation by sunlight (Facino et al., 1995). When *Echinacea* has been investigated *in vivo* in the presence of active infectious disease, it has provided protection against a number of pathogens including *Lysteria monocytogens* and *Candida albicans* (Roesler et al., 1991a; Steinmüller et al., 1993). Other researchers have shown that *Echinacea* mixed with vitamin C was able to reduce the severity and duration of the common cold in humans (Scaglione et al., 1995).

Taken together, these data suggest that *Echinacea* may have potential in stimulating the immune system response of livestock under stressful husbandry conditions.

EQUINE RESEARCH

Two studies have been identified that seek to quantify the effect of *Echinacea* on horses. The first report in the literature appeared in 1994 in a German-language case study (May 1994). This article reported a case of two horses suffering from strangles. Both horses were initially treated with an unidentified drug, in order to prevent the spread of the disease to other animals in the barn. After an undisclosed period of time, both horses were given an *Echinacea* composite for 2 days, and within 24 hours both horses made a marked recovery. Interpretation of this study is difficult due to the absence of detailed product characterization, dose, or time frame. However, it does mark the first time that such a report has appeared in the literature.

A more recent report (O'Neill et al., 2002) describes a study in which the effect of a standardized *Echinacea* extract was quantified in healthy horses. This study used an aqueous extract of *Echinacea angustifolia* prepared from powdered root that was standardized to 4% echinacoside (a marker of plant maturity and potency). The trial involved eight horses, each of which was placed on the extract for 42 days, or for 42 days on an inactive placebo. Blood samples were taken every 7 days,

and were subjected to a complete hematology and biochemistry screen, and a phagocytic function test. With respect to immune system effects, this study suggested that *Echinacea* has similar effects in horses as in mice, and stimulates neutrophils to increase their phagocytic function (Figure 19.1). In addition, *Echinacea* increased the production of lymphocytes (Figure 19.2) and decreased the levels of circulating neutrophils in the blood presumably by increasing membrane permeability and migration into tissues. All of these effects on the immune system have been described in research reports in other species. However, the hematology profiles in this study characterized an unexpected effect of the *Echinacea* extract on the oxygen-transport cells (red blood cells). There was a significant increase in the number and size of red blood cells (Figure 19.3), and a significant increase in the level of hemoglobin (the molecule responsible for transporting oxygen) in the blood (Figure 19.4). This effect of *Echinacea* has not been reported in previous studies of any species, and may be of particular interest to those involved with performance horses.

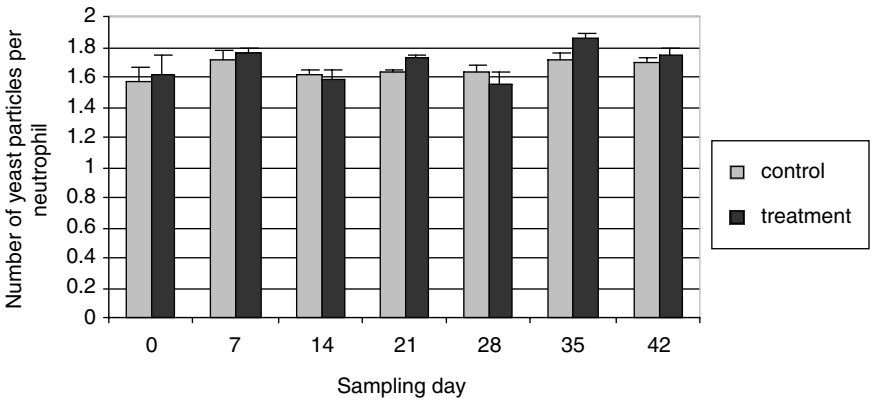

FIGURE 19.1 Mean number of ingested cells per neutrophil with and without treatment with *Echinacea*. The mean number of yeast cells ingested per neutrophil was statistically different between treated and control neutrophils on Day 21 ($p < 0.05$) and Day 35 ($p < 0.05$). (From O'Neil et al., 2002, *Equine Vet. J.* 34: 222–227. With permission.)

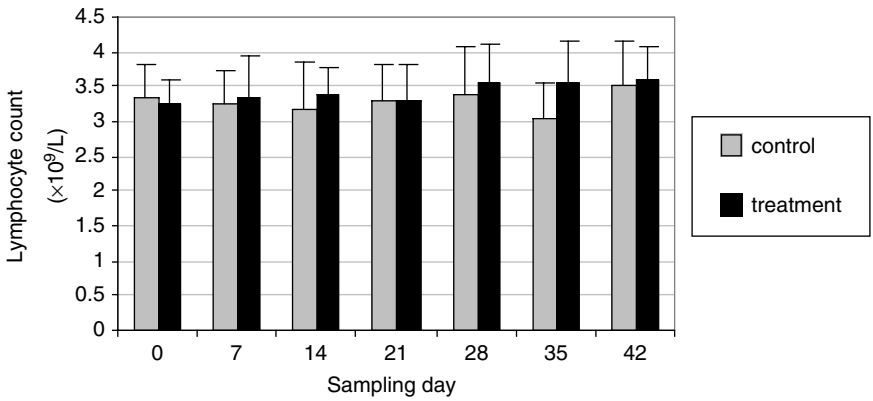

FIGURE 19.2 Mean lymphocyte count. The mean lymphocyte count after treatment with *Echinacea* was significantly greater than that of control on Day 35 ($p < 0.01$). (From O'Neil et al., 2002, *Equine Vet. J.* 34: 222–227. With permission.)

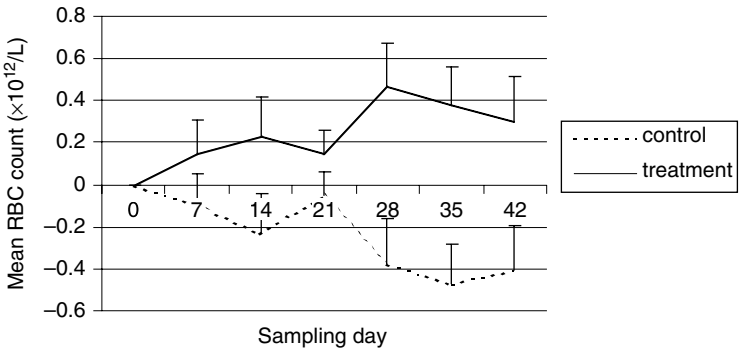

FIGURE 19.3 Mean change in red blood cell count. The mean change over time in red blood cell count (RBC) from Day 0 to 42 was significantly different between horses that were treated with *Echinacea* vs. controls ($p < 0.01$). (From O'Neil et al., 2002, *Equine Vet. J.* 34: 222–227. With permission.)

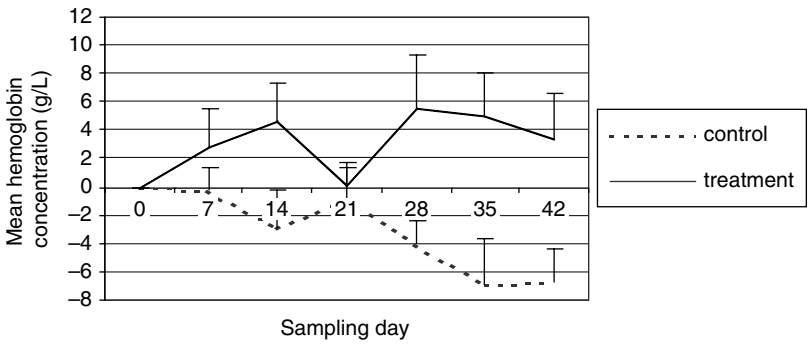

FIGURE 19.4 Mean change in hemoglobin concentration. The mean change in hemoglobin from Day 0 to 42 was significantly different between treated and control horses ($p < 0.05$). (From O'Neil et al., 2002, *Equine Vet. J.* 34: 222–227. With permission.)

BOVINE RESEARCH

A very recent report in the literature describes the immunological effects of a commercial product containing *Echinacea purpurea* on bovine leucocytes (Schuberth et al., 2002). Bovine mononuclear (MNC) and polymorphonuclear (PMN; predominately neutrophils) cells were isolated from cows and cultured for up to 44 hours in the presence or absence of an extract of the product and its individual components. Flow cytometry techniques were utilized to characterize the effect on the size, morphology, and function of the cells. None of the individual components, which included *Thuja occidentalis, Echinacea* sp. and elemental phosphorus, had any substantial effect on MNC. However, *Echinacea* alone was found to reduce the size of the PMNs, with a concurrent increase in cell viability after 20 hours of culture. *Echinacea* was also able to enhance the ability of the PMNs to kill target cells via antibody-independent cytotoxicity. This *in vitro* study provides basic evidence for the immunomodulatory potential of *Echinacea* in bovines. However, conclusions to be drawn from this study are limited, as the study cannot take into account the considerable metabolism that *Echinacea* itself undergoes *in vivo*.

Another recent *in vivo* report describes a pilot investigation into the effects of an *Echinacea* extract on dairy calves (O'Neill et al., unpublished data, 2002). The standardized *Echinacea* extract was the same as that used in the recent equine research report, as described above (O'Neill et al.,

2002). Eight dairy heifers between the ages of 2 and 52 days participated in the 14-day trial. Calves were fed 5 ml of *Echinacea* extract per liter of milk per day. Measurement parameters included complete hematology and biochemistry screens, and the incidence of pneumonia, scour, and leg infections. Over the 14 days, there was a trend to an increase in neutrophils and total plasma protein. There were no significant effects on the incidence of scour or pneumonia. However, this study is limited by its short time frame, and statistically significant results may have resulted from a longer supplementation schedule, as was found in the previous equine study (O'Neill et al., 2002).

POULTRY RESEARCH

There is a paucity of research involving *Echinacea* and chickens. A German-language publication describes an *in vivo* study comparing the effect of a drug complex containing 30% *Echinacea angustifolia* extract, and pure *E. angustifolia* extract in the humoral immune response of intact and immunodeficient chickens (Schranner et al., 1989). Investigators administered the experimental extracts in two oral doses, and measured immunoglobulin and antibody production in response to human serum albumin injections. In normal chickens, the administration of the complex drug resulted in a rise in serum immunoglobulin concentration, as well as an increase in the three classes of antibodies. In immunodeficient chickens, the complex drug caused a slight production of IgG. However, there was no significant difference in humoral immune parameters when pure *E. angustifolia* extract was fed.

SWINE RESEARCH

A series of research projects concerning *Echinacea* use in swine were conducted at the Iowa State University (ISU) Swine Nutrition and Management Center in temperature-regulated nursery rooms (Holden and McKean, 2002). The purpose of these studies was to compare the health and performance of weanling pigs fed varying levels of *Echinacea* with those receiving a subtherapeutic level of a common antibiotic (Mecadox). Weanling pigs were randomly allocated to 20 or 24 pens of five pigs each, providing four to six replications of the dietary treatments. Pigs were weighed, and feed disappearance was measured weekly for 5 weeks. In the first year of studies (1997), the project was completed at the end of the nursery phase. When the studies were repeated in 1999–2000, postnursery weights were recorded every 4 weeks to evaluate long-term effects of the nursery treatments. Average daily gain (ADG), average daily feed (ADF), and feed efficiency (F/G) were analyzed with the pen as the experimental unit. Where appropriate, one pig at the end of the nursery phase from each botanical treatment pen was taken to the ISU Meat Laboratory, slaughtered, and various muscles evaluated for sensory and quality characteristics. Pigs fed Mecadox were not evaluated because of a 42-day withdrawal requirement.

At the tested inclusion levels (0.1%, 0.5%, and 2.0%), no statistical advantage existed when compared with the diet containing 45 ppm of Mecadox or with a "negative" control containing no antimicrobial or botanical inclusions (Table 19.1). *Echinacea*-treated pigs exhibited a slight, but not objectionable, off-flavor of their meat when compared to pigs fed noninclusion levels. The study noted that in Weeks 0 to 3 and 0 to 4 the higher levels of *Echinacea* (0.5% and 2.0%) were significantly more efficient ($p < 0.05$), but ADG and ADF were not statistically different. Total performance for the entire experiment, Weeks 0 to 5, was not statistically different. These data suggest higher levels of *Echinacea* enhanced F/G compared to the 0% *Echinacea* during the first 2 weeks and were greater than the Mecadox diet during the Weeks 0 to 3 and 0 to 4. Overall, performance was similar, suggesting minimal subclinical stress during this experiment. Higher levels of *Echinacea* may be required to enhance growth rate and feed efficiency.

Lower levels of *Echinacea* (0%, 0.10%, 0.25%, and 0.50%) did not enhance any performance parameters, while higher levels (3.0%) of *Echinacea* enhanced overall ADG in the Week 0 to 5

TABLE 19.1
Effect of *Echinacea*

	Mecadox	*Echinacea*%			
		0.0	**0.1**	**0.5**	**2.0**
		Week 0–2			
ADG, kg	0.20	0.16	0.17	0.18	0.17
ADF, kg	0.33	0.31	0.29	0.29	0.29
F/G[ab]	1.62	1.93	1.71	1.62	1.65
		Week 0–3			
ADG, kg	0.25	0.22	0.23	0.24	0.24
ADF, kg	0.41	0.39	0.38	0.38	0.39
F/G[c]	1.66	1.79	1.65	1.57	1.59
		Week 0–4			
ADG, kg	0.32	0.29	0.30	0.30	0.31
ADF, kg	0.51	0.49	0.48	0.48	0.50
F/G[d]	1.60	1.71	1.62	1.58	1.58
		Week 0–5			
ADG, kg	0.38	0.35	0.35	0.36	0.37
ADF, kg	0.64	0.61	0.59	0.60	0.61
F/G	1.65	1.73	1.68	1.65	1.66

[a] Mecadox vs. 0.0%, $p < 0.05$.
[b] $p < 0.05$; vs. 0.5% and 2.0%, $p < 0.01$.
[c] 0.0% vs. 0.5% and 2.0%, $p < 0.05$.
[d] vs. 0.5% and 2.0%, $p < 0.02$.

nursery period when compared to 0% and 1.5% levels, and supported gains equal to the Mecadox diet.

This series of experiments is an important contribution to the database of veterinary applications of *Echinacea*, as it specifically compares *Echinacea* application with that of subtherapeutic antibiotics. Results from these experiments suggest that *Echinacea* may be a good substitute for antibiotics in feed, and results in equal or better performance parameters. It would be of great value to repeat these studies with an *Echinacea* product that is both standardized and characterized, in order to enhance repeatability by other investigators.

Earlier work by German scientists describes field studies on the effectiveness of an herbal composite containing *Echinacea* sp. (Both, 1987) in treating and preventing mastitis-metritis-agalactia syndrome. This study was conducted over a 6-year period on slightly fewer than 10,000 farrowings in 65 herds. The incidence of the syndrome was significantly reduced by parenteral administration of the drug, as was the incidence of scour in the neonatal piglets. Eight of the 65 herds did not respond to the treatment, and this was considered to be due to poor on-farm hygiene, inadequate nutrition, and the age of the sows. This work is an important long-term study of herbs in preventing and treating swine diseases. However, it has the obvious disadvantage of looking at a composite, making individual assessment of the components impossible.

TOXICITY RESEARCH

The toxicity of *Echinacea* sp. appears to be very low. Researchers have performed acute, subacute, and genotoxicity studies on mice and rats and found *E. purpurea* to be "virtually non-toxic to rats

and mice" (Mengs et al., 1991). Test animals were given oral doses of the expressed juice over a 4-week period at a dose equivalent to many times the human therapeutic dose. Laboratory tests and necropsy findings could not demonstrate any evidence for toxicity. All mutagenicity and carcinogenicity studies gave negative results. In a comprehensive review of the literature on the safety of *E. purpurea*, Parnham (1996) concluded that the squeezed sap of the plant is well tolerated in long-term use, with no significant side effects when the sap was administered orally. This conclusion is echoed by Hobbs (1994), who found no published reports indicating that *Echinacea* had toxic side effects. In a recent *in vitro* study examining the efficacy of *Echinacea* (See et al., 1997), *Echinacea* extract was not found to diminish the viability of peripheral blood mononuclear cells after 4 hours at concentrations up to 1000 g/mL.

A study by Röder (1994) found *E. purpurea* to contain pyrrolizidine alkaloids at a level of 0.006%. Unsaturated pyrrolizidine alkaloids are known to be hepatoxic in animals and humans (Pearson, 2000). However, the pyrrolizidine alkaloids found in *Echinacea* (isotussilagine and tussilage) have a saturated pyrrolizidine nucleus and are not thought to be toxic (Newall et al., 1996).

Echinacea has been shown to inhibit enzyme activity in human sperm at high concentrations *in vitro* (Ondrizek et al., 1999a). High concentrations have also been demonstrated to reduce oocyte penetration by sperm, and to cause denaturation of sperm DNA (Ondrizek et al., 1999b). Long-term exposure of the cells to *Echinacea* caused DNA denaturation and decreased sperm viability, even at low concentrations (Ondrizek et al., 1999b). These data suggest caution when feeding *Echinacea* to breeding livestock.

These toxicity data demonstrate that *Echinacea* is safe when fed to laboratory animals. None of the species-specific research was able to identify any possible side effects of the treatment across the duration of the study. However, no acute or chronic studies have been published confirming safety in livestock species.

SUMMARY

A new landscape of animal husbandry, and in particular the movement away from antibiotics in livestock feed, has created a whole new incentive and urgency to quantifying the usefulness of botanicals in animal diets. *Echinacea* has been widely researched in laboratory animals for its potential clinical uses. The toxicity of *Echinacea* is reported to be very low. The only toxic response identified is an ability to inhibit the viability and function of sperm, which is of particular concern to those raising livestock for breeding. Research in horses, cattle, and swine has been reported, which provides some rationale for the use of this botanical in livestock feed. In horses, *Echinacea* extract has reduced infections of strangles, and stimulated immune and oxygen-transport cells. Cattle research has shown that supplementation with *Echinacea* can stimulate the phagocytic function of bovine polymorphonuclear cells. Finally, a series of swine studies demonstrated that *Echinacea* could improve performance parameters in nursery pigs to a level not statistically different from a common antibiotic. The research reports available suggest that *Echinacea* can be a rational inclusion into livestock husbandry practices under appropriate conditions, and may provide an effective alternative to subtherapeutic antibiotics.

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Biological Sciences

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