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Echinacea in infection1–4

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ABSTRACT

Ongoing studies have developed strategies for identifying key bioactive compounds and chemical profiles in *Echinacea* with the goal of improving its human health benefits. Antiviral and antiinflammatory–antipain assays have targeted various classes of chemicals responsible for these activities. Analysis of polar fractions of *E. purpurea* extracts showed the presence of antiviral activity, with evidence suggesting that polyphenolic compounds other than the known HIV inhibitor, cichoric acid, may be involved. Antiinflammatory activity differed by species, with *E. sanguinea* having the greatest activity and *E. angustifolia, E. pallida,* and *E. simulata* having somewhat less. Fractionation and studies with pure compounds indicate that this activity is explained, at least in part, by the alkamide constituents. Ethanol extracts from *Echinacea* roots had potent activity as novel agonists of TRPV1, a mammalian pain receptor reported as an integrator of inflammatory pain and hyperalgesia and a prime therapeutic target for analgesic and antiinflammatory drugs. One fraction from *E. purpurea* ethanol extract was bioactive in this system. Interestingly, the antiinflammatory compounds identified to inhibit prostaglandin E_2 production differed from those involved in TRPV1 receptor activation. *Am J Clin Nutr* 2008;87(suppl):488S–92S.

KEY WORDS Alkamide, antiinflammatory, antipain, antiviral, cichoric acid, *Echinacea*, *Echinacea angustifolia*, *Echinacea pallida*, *Echinacea purpurea*, *Echinacea sanguinea*, *Echinacea simulate*, HIV, TRPV1 receptor

CENTER ORGANIZATION

The primary goal of the Iowa Center for Research on Botanical Dietary Supplements is to improve our understanding of the characteristics of *Echinacea, Hypericum*, and *Prunella* that contribute to human health and thereby pave the way for optimizing these supplements for study in future clinical trials. Our center focuses on infection with an emphasis on antiviral, antiinflammatory, and antipain activities. This article summarizes some of our work on *Echinacea*.

A central strategy of the Iowa center has been to use biological diversity to help to identify active constituents and determine mechanisms of action. It is tempting to consider the diversity of these plant genera and the complexity of their constituents as barriers to understanding their potential health benefits. However, the range of variation in these plants, when systematically analyzed, provides a strong foundation on which to develop the strategies and tools needed to produce the most efficacious products for a growing body of consumers.

The center is organized into 3 cores: Germplasm and Phytochemical Profiling; Separations, Structure, Bioavailability; and Administration, Data Management, Statistics, and Bioinformatics. Three projects are supported: defining antiviral activities in *Echinacea*, *Hypericum*, and *Prunella* species; antiinflammatory activity of *Echinacea*, *Hypericum*, and *Prunella* species; and pain-receptor-mediated antiinflammatory activity of *Echinacea* and *Hypericum* species.

PRODUCTION OF WELL-CHARACTERIZED PLANT MATERIAL

A unique resource of the center is our collection of a genetically diverse set of well-documented plant populations of *Echinacea* and the conservation of these genetic stocks at the US Department of Agriculture-Agricultural Research Service North Central Regional Plant Introduction Station (NCRPIS). This resource gives us strength in controlling the production of plant materials and links with our expertise in genomic analysis and broad-based plant metabolic profiling and our ability to integrate complex datasets by using bioinformatics and other statistical tools. The NCRPIS, which is located at Iowa State University, is one of the main active gene banks in the US National Plant Germplasm System, and it conserves extensive collections of

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known-source medicinal plants, with an emphasis on *Echinacea* and *Hypericum*.

Since the late 1990s, the NCRPIS has acquired >150 distinct wild populations (or accessions) of *Echinacea*, representing all recognized species and varieties from throughout their ranges, including 2 federally endangered taxa, *E. laevigata* and *E. tennesseensis*. The genetic integrity of these populations is preserved by regenerating seed samples under controlled conditions in screened field cages with insect pollinators, typically honeybees (1). To ensure unbiased sampling of *Echinacea* populations for regeneration, research has been directed toward understanding seed dormancy and methods to overcome it (2, 3).

During seed regeneration, taxonomic identities are verified and populations are characterized for phenotypic traits with a standardized descriptor list. Phenotypic descriptors along with images and detailed passport data describing each accession are available from the Germplasm Resources Information Network database (4). Seeds of all available accessions are distributed for research and educational purposes at no cost to the user.

Echinacea samples used by center researchers have typically been produced from NCRPIS accessions. By using these accessions produced under known conditions, we minimize both the genetic and the environmental components of biochemical variation in the resulting products, which increases the overall repeatability of bioassays. Long-term, replicated field plantings of the 3 primary medicinal species, *E. angustifolia*, *E. pallida*, and *E. purpurea*, were established in 2003 to optimize root production and determine the effects of disease and shading on plant survival and productivity. Dried roots from these plantings have been a major source of plant material for our research projects. Additional accessions have also been supplied to researchers as root samples from plants used for seed regeneration, after successful completion of the seed-production process, and as leaf and pollen samples. After production and processing, all plant samples for center use are inventoried with a standardized coding system, and unextracted dried plant materials are packaged in nitrogen and stored frozen at −20 °C. In addition to the supply of plant materials for bioassays, a carefully selected, diverse array of ≈40 *Echinacea* accessions is being characterized biochemically for alkamides and caffeic-acid derivatives and genetically for both nuclear and chloroplast DNA variation. These characterization data should be valuable for describing the extent of chemical variation, elucidating taxonomic relations, and providing a framework for phylogenetic analysis and future bioassay.

ANTI-HIV ACTIVITIES OF *ECHINACEA*

The antiviral activities of *Echinacea* extracts and its metabolic constituents are surprisingly poorly studied. A recent, prominent study identifying a lack of efficacy of *E. angustifolia* extracts against rhinovirus infection has discouraged additional clinical antiviral studies on botanical extracts (5). However, recent in vitro studies within our center identifying antiviral activities in *Echinacea* against HIV are promising. Constituents responsible for the anti-HIV activity in our plant extracts were identified through bioactivity-driven fractionation studies. A example of our success with this approach is shown here for *E. purpurea.*

Initially, extracts from 6 *Echinacea* species were tested for inhibition of replication of the HIV molecular clone pNL4-3. All HIV infectivity assays were performed in the HeLa37 cell line that expresses the viral receptor and co-receptors (6). Known titers of HIV were added to cells in the presence of the botanical extract. Cells were fixed and immunostained for viral antigens at 40 h after infection. Cells immunopositive for HIV were represented as percentage of control values. Extracts from *E. purpurea* consistently provided the most robust inhibition of HIV with little or no cellular cytotoxicity. Increasing concentrations of *E. purpurea* extract had antiviral activity with a 50% inhibitory concentration (IC50) of 2.4 μg/mL (**Figure 1**). This species contains the anti-HIV compound cichoric acid and thus was anticipated to inhibit HIV replication (7). To determine whether constituents other than cichoric acid had antiviral activity, *E. purpurea* extracts were fractionated. Seven fractions were generated; no fraction had detectable cytotoxicity or endotoxin contamination (**Figure 2**A). Fraction 1, the most polar, had significant anti-HIV activity; the other fractions had no antiviral activity. HPLC analysis of fraction 1 documented that a series of caffeic acid derivatives and other polyphenolics including cichoric acid were present (Figure 2B). Subfractionation into 9 subfractions yielded 6 with anti-HIV activity (**Figure 3**); HPLC analysis of these subfractions showed that each fraction was composed of different constituents. Subfractions 1–6 all contained numerous constituents including cichoric acid; subfractions 1–3, 1–4, 1–7, and 1–8 exhibited some antiviral activity and contained multiple constituents that absorbed light at 330 nm and likely are polyphenolics. Interestingly, subfraction 5 absorbed light at 254 nm but not at 330 nm and may contain glycosylated flavanoids. We anticipate that we will successfully continue to use this bioactivity-driven fractionation approach to identify the botanical compounds responsible for the antiviral activity.

ANTIINFLAMMATORY SCREENING USING RAW264.7 MACROPHAGES

The antiinflammatory activity of *Echinacea* extracts, fractions, and constituents was assessed with RAW264.7 cells treated with and without lipopolysaccharide, and prostaglandin E_2 (PGE_2) accumulation was measured. This widely used screen for antiinflammatory activity is based on PGE_2 production arising from cyclooxygenase-1 and -2 activation, a key event in inflammation. Initial studies showed that soxhlet ethanol extracts of *Echinacea* provided the greatest antiinflammatory activity. Activity of soxhlet extracts of *E. purpurea, E. angustifolia, E. pallida, E. tennesseensis*, *E. simulata*, and *E. sanguinea* at 15 μg/mL, which were harvested during the fall of 2003, 2004, and 2005, did not differ significantly by repeat extraction or harvest.

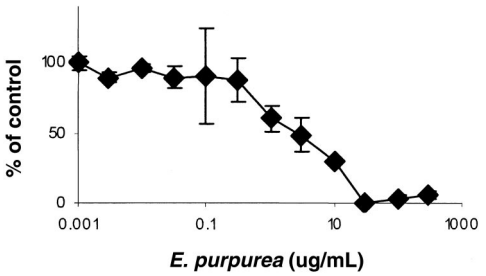

FIGURE 1. Ability of *Echinacea purpurea* ethanol extract (PI621307) to inhibit HIV replication. Data shown represent the mean ± SEM for each data point from 3 different experiments with triplicate samples in each experiment.

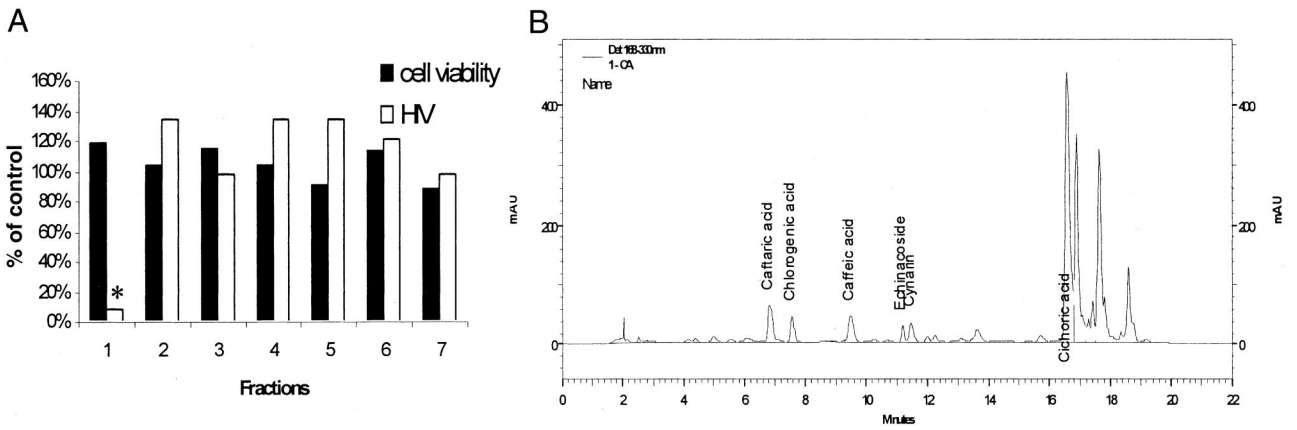

FIGURE 2. Inhibition of HIV infectivity by *Echinacea purpurea* fractions. (A) Ability of 7 HPLC-separated fractions (100 μg/mL) to inhibit HIV infection (white bars) or cytotoxicity (black bars). Each bar represents the mean of triplicates. The antiviral activity found in fraction 1 that is highlighted with an asterisk showed efficacy against HIV-1 as determined by a Student's *t* test comparing the untreated and treated samples ($P = 0.0001$). (B) HPLC analysis of *E. purpurea* fraction 1. Phenolic acid standards were run to allow identification of compounds.

E. sanguinea inhibited PGE_2 production to the greatest extent; *E. angustifolia, E. pallida*, and *E. simulata* inhibited less; and *E. tennesseensis* and *E. purpurea* extracts were not inhibitory at this concentration (**Figure 4**) but were at higher concentrations (8). Concentrations of alkamides and ketones in these extracts were determined, but these compounds did not simplistically explain bioactivity. For example, *E. angustifolia* and *E. purpurea* were rich in Bauer Amide 8, which was not abundant in *E. pallida.* There was relatively little cytotoxicity of *Echinacea* even at doses 10-fold higher than those assayed for antiinflammatory activity. All extracts reported here were negative for endotoxin contamination.

HPLC fractions of *E. pallida* and *E. angustifolia* had similar patterns of effects on PGE_2 production, with the strongest inhibition in fraction 3 for both species. Interestingly, the more polar fractions (fractions 1 and 2 containing polyphenols such as caffeic acid and Bauer alkamides 1–7) had less antiinflammatory activity at higher concentrations, but fraction 3, which contained abundant alkamides (unique in this fraction for *E. purpurea* and *E. angustifolia* were Bauer amides 8 and 9), significantly reduced

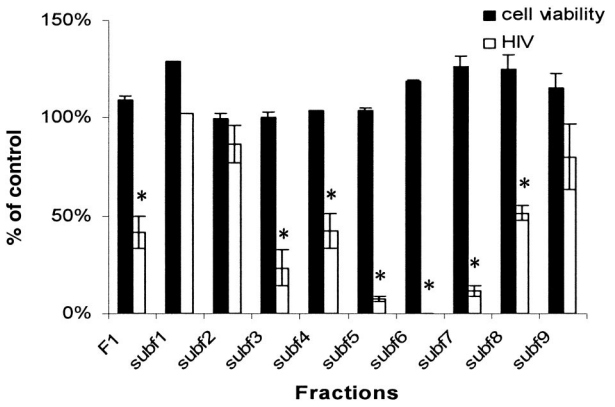

FIGURE 3. Antiviral activity and cytotoxicity associated with subfractions 1-9 from *Echinacea purpurea* fraction 1. All HIV infectivity (white bars) and cytotoxicity (black bars) assays were performed with 100 μg/mL of the fraction or subfraction. Values represent the mean ± SEM for 2 experiments performed in triplicate. Asterisks represent findings that are significantly different from control infections as determined by a Student's *t* test ($P = 0.0001$).

LPS-induced PGE_2 production (**Figure 5**). Our observations are consistent with observations of others showing antiinflammatory activity with alkamide extracts containing Bauer alkamides 8 or 9 (9). However, because *E. pallida* does not contain amides 8 or 9; other compounds, possibly ketones, must contribute the activity.

Numerous alkamides synthesized by our center were tested for antiinflammatory activity, and all the synthesized alkamides (ie, Bauer 2, 8, 10, 11, 13, and 14) screened to date significantly inhibited the production of PGE_2 ($P < 0.001$) at 50 μmol/L (8). Only Bauer amide 14 significantly inhibited PGE_2 production at 10 μmol/L ($P < 0.05$), although amides 8 and 12 at 10 μmol/L inhibited at $P < 0.08$. From these data, amide 14 is the only synthesized alkamide that significantly reduced PGE_2 at all concentrations screened. These data suggest that the alkamides in *Echinacea* are important for its antiinflammatory activity. Amide 8 may play a key role because of its abundance in *E. angustifolia* fraction 3, in which PGE_2 production by LPS-treated RAW264.7 cells was potently reduced (Figure 5).

Subfractionation of *E. angustifolia* fraction 3 showed that the less polar subfractions have antiinflammatory activity, with subfractions 3D and 3E exhibiting the greatest reduction in PGE_2 accumulation (data not shown). Alkamides are present in subfractions 3B-E: 3D contains alkamides 5, 8, 9, and 14; 3E contains 10 and 11; 3B contains alkamide 1; and 3C contains alkamides 1, 2, 3, 5, 12, 13, and 14.

This series of studies suggests that *Echinacea* alkamides may contribute to observed antiinflammatory activity because they are readily identified in the active extracts and exhibit appreciable activity. However, results with purified alkamides indicate that single compounds failed to completely account for antiinflammatory activity and, thus, may interact with each other or with other compounds to explain the observed antiinflammatory activity.

MEDIATION OF PAIN-RECEPTOR ACTIVITY BY *ECHINACEA*

TRPV1 in pain and inflammation

TRPV1 (transient receptor potential channel, vanilloid subfamily member 1, VR1) (10) is a ligand-gated cation channel pain

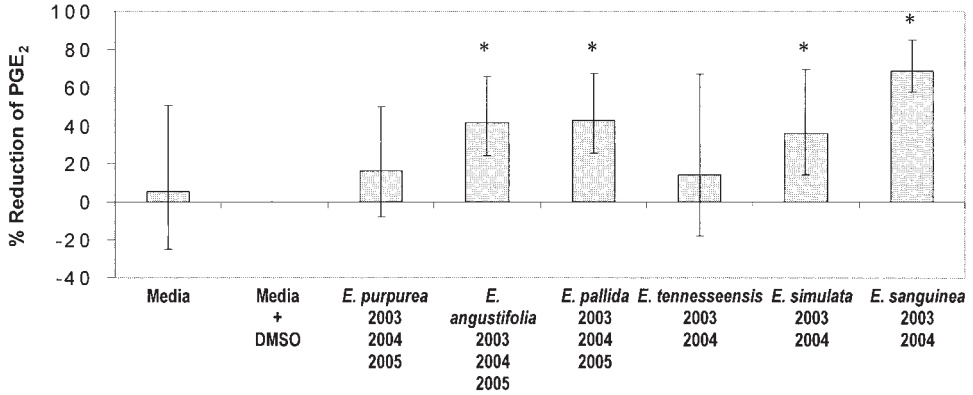

FIGURE 4. Extracts of *Echinacea* species from different harvest dates were studied at 15 μg/mL for their effects on prostaglandin E_2 (PGE_2) production. Data (originally ng/mL PGE_2) were normalized to the DMSO-lipopolysaccharide (LPS)–treated control and are presented as means ± 95% CIs. *E. angustifolia, E. pallida,* and *E. sanguinea* ($P < 0.001$) reduced PGE_2 production compared with medium + DMSO controls. Data shown are with LPS; studies were conducted without LPS, but PGE_2 values were not altered by *Echinacea*. $^{*}P < 0.05$ compared with medium + LPS + DMSO in a Dunnett multiple-comparison test. Redrawn from reference (8).

receptor. It was initially cloned by using capsaicin, the potent compound of hot peppers, which is a strong ligand (11). TRP channels are extremely nonselective; TRPV1 is activated by factors including capsaicin, protons, noxious heat (>42 °C), the endocannabinoid anandamide, lipoxygenase product, and ethanol (12). It is considered to be a key integrator of external stimuli, both chemical and physical, into the common signal of inward ion currents (11). Receptors from the TRP channel family, as well as other classes of receptors such as CB, ASIC, and Trek-1, are predominantly expressed in sensory tissues, such as nociceptors and skin. These receptors are thought to provide feedback to sense, transmit, and integrate pain and associated inflammatory responses (11). Growing evidence indicates that TRPV1 acts as an integrator of inflammatory pain and hyperalgesia, making it an excellent potential target for analgesic or antiinflammatory agents. Because of its importance in pain and inflammatory responses, TRPV1 protein structure-activity relations are being analyzed extensively to determine which TRPV1 sites are important for interaction with each of its ligands. TRPV1 is also desensitized by its ligands. Desensitization of TRPV1 after its activation in these neurons is crucial in blocking pain transmission (11). Therefore, compounds that serve as agonists have potential use as analgesics or antiinflammatory agents. In addition, capsaicin can produce a hypotensive effect in spontaneously hypertensive rats, indicating that activation of TRPV1 may contribute to the treatment of hypertension (13).

Echinacea species comparison

Because of reported effects on inflammatory pain, we investigated the effects of *Echinacea* extracts on TRPV1-dependent inward ion currents by using transient expression of TRPV1 in frog oocytes (11). In this system, TRPV1 cRNA is injected into healthy oocytes and is expressed transiently; then the TRPV1-expressing oocytes are bathed in *Echinacea* extracts or capsaicin for 10 s. Whole-cell currents are recorded by a 2-electrode voltage clamp recording system. Extracts of roots of *E. angustifolia* evoked a current 10-fold greater than a saturating dose of capsaicin (**Figure 6**). *Echinacea* extracts rapidly desensitize the TRPV1 channel even in the absence of added calcium, which suggests that TRPV1 activation may occur through a mechanism different from that of capsaicin. Leaf and flower extracts also activated TRPV1.

Identification of bioactive constituents

Aqueous extracts of *Echinacea* contain unusual polysaccharides reported to be responsible for certain antiinflammatory activities (14). When *Echinacea* is extracted with solvent containing <90% water, polysaccharides are absent; thus, our ethanol extracts would presumably not contain them. To directly test whether *Echinacea* polysaccharides activate the TVPR1 channel, we prepared water extracts of *E. purpurea*, *E. angustifolia*, and *E. pallida* and tested them with the frog-oocyte model.

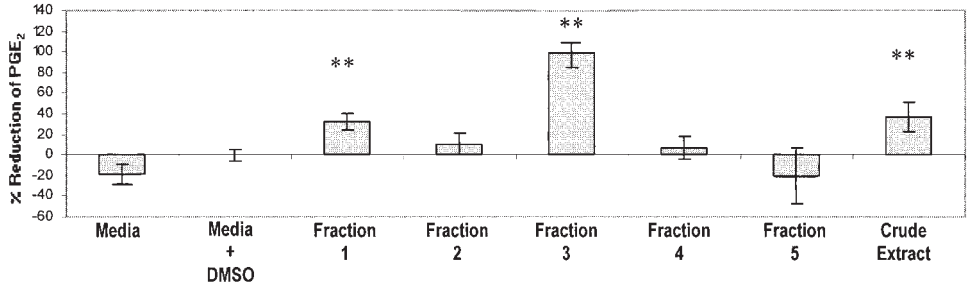

FIGURE 5. Preparative HPLC fractions of *Echinacea angustifolia* soxhlet ethanol extract were assessed for reduction of prostaglandin E_2 (PGE_2) production and are presented as means ± 95% CIs. All treatments are shown with lipopolysaccharide (LPS) because the fractions did not alter PGE_2 production in the absence of LPS. Fractions 1 and 3 significantly reduced LPS-induced PGE_2 production. $n = 3$. $^{**}P < 0.05$, representative of a Dunnett multiple-comparison test.

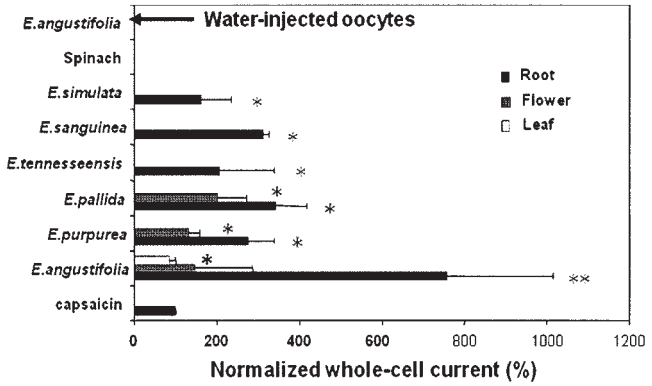

FIGURE 6. Relative potency of 70% ethanol *Echinacea* extracts (0.25 mg/mL) on TRPV1 (mean ± SEM, $n \geq 4$ independent oocytes for each value). Current responses were normalized in each cell to responses obtained with capsaicin (10 μmol/L). Spinach extract (0.25 mg/mL) was used as the control. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different when compared with spinach control (Student's *t* test). Extracts evoked no responses in water-injected cells.

Echinacea aqueous extracts did not activate TRPV1, which indicates that the polysaccharides do not appear to be TRPV1 ligands. Because several bioactivities, including cannabinoid CB pain-receptor activation (15) and antiinflammatory effects (described here), can be attributed at least in part to the alkamide constituents of *Echinacea*, we also tested the effect of 6 purified *Echinacea* alkamides on TRPV1 with use of the frog oocyte model. Interestingly, no alkamide tested activated the TRPV1 channel. We are now using a combination of subfractionation of *Echinacea* extracts and bioassays to identify the bioactive constituents. CB and TRP receptors appear to be intimately related. CB1 stimulation modulates TRPV1 activities in cultured rat dorsal root ganglion cells, and CB1 and TRPV1 receptors are highly coexpressed in nociceptive primary sensory neurons (15).

FUTURE OBJECTIVES

Our center's overarching objectives include identifying compounds contributing to the antiviral, antiinflammatory, and pain-control effects of *Echinacea* and to *Echinacea* toxicity. We further seek to assess the influence of plant species and population on bioactive constituents and to understand their mechanisms of action, in particular, the cellular signaling pathways and critical receptors and the effects of their interactions. We will assess the bioavailability of key constituents of *Echinacea* supplements to fill this important gap, because bioavailability likely plays a key role in translating the results of bioassays to potential human health effects.

The contributions of the authors were as follows—DFB: directs the Center and supervised the research of CAL; MPW: co-leads the Germplasm and Phytochemistry Core; CAL: conducted the studies reported in Figures 4 and 5; LW: conducted the studies reported in Figure 6; JB: synthesized alkamides; AKSS: conducted fractionation of *Echinacea*; GAK: supervised the research of JB; PAM: supervised the research of AKSS; ESW: supervised the research of LW; QL: instructed the Wurtele laboratory in the oocyte TRVP1 studies; SCH: consulted with the Wurtele laboratory in the interpretation of the oocyte TRVP1 studies; WJM: supervised the research of JPP; JPP: conducted the studies reported in Figures 1, 2, and 3.

DFB served on the National Toxicology Program (NIEHS) Board of Scientific Counselors during most of the time that this research was being conducted. The remaining authors had no financial or personal interests in any company or organization sponsoring the research, including advisory board affiliations.

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