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Echinacea Species and Alkamides Inhibit Prostaglandin E_2 Production in RAW264.7 Mouse Macrophage Cells

CARLIE A. LALONE,[†,‡,§] KIMBERLY D. P. HAMMER,[†,‡,§] LANKUN WU,[†,#] JAEHOON BAE,[†,||] NORMA LEYVA,[†,⊥] YI LIU,[†,§] AVERY K. S. SOLCO,[†,§] GEORGE A. KRAUS,[†,||] PATRICIA A. MURPHY,[†,§] EVE S. WURTELE,[†,#] OK-KYUNG KIM,[†,⊗] KWON II SEO,[†,△] MARK P. WIDRLECHNER,[†,×,◇] AND DIANE F. BIRT*[,†,‡,§]

The Center for Research on Dietary Botanical Supplements, Interdepartmental Genetics Graduate Program, Department of Genetics, Development, and Cell Biology, Department of Chemistry, Department of Horticulture, Department of Agronomy, Department of Statistics, and Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011

Inhibition of prostaglandin E_2 (PGE_2) production in lipopolysaccharide-stimulated RAW264.7 mouse macrophage cells was assessed with an enzyme immunoassay following treatments with *Echinacea* extracts or synthesized alkamides. Results indicated that ethanol extracts diluted in media to a concentration of 15 μg/mL from *E. angustifolia*, *E. pallida, E. simulata*, and *E. sanguinea* significantly inhibited PGE_2 production. In further studies, PGE_2 production was significantly reduced by all synthesized alkamides assayed at 50 μM, by Bauer alkamides 8, 12A analogue, and 14, Chen alkamide 2, and Chen alkamide 2 analogue at 25 μM and by Bauer alkamide 14 at 10 μM. Cytotoxicity did not play a role in the noted reduction of PGE_2 production in either the *Echinacea* extracts or synthesized alkamides. High-performance liquid chromatography analysis identified individual alkamides present at concentrations below 2.8 μM in the extracts from the six *Echinacea* species (15 μg/mL crude extract). Because active extracts contained <2.8 μM of specific alkamide and the results showed that synthetic alkamides must have a minimum concentration of 10 μM to inhibit PGE_2, it is likely that alkamides may contribute toward the anti-inflammatory activity of *Echinacea* in a synergistic or additive manner.

KEYWORDS: ***Echinacea purpurea*; *Echinacea angustifolia*; *Echinacea pallida*; *Echinacea tennesseensis*; *Echinacea simulata*; *Echinacea sanguinea*; anti-inflammatory; cytotoxicity**

INTRODUCTION

The use of *Echinacea* as a medicinal herb is prominent in the United States, with sales encompassing approximately 10% of the total U.S. market in botanical supplements (*1*). With the increasing popularity of *Echinacea*, it is important to identify its active constituents and determine extraction methods that yield the proper doses of active constituents to elicit the desired medicinal effect. Three species, *E. purpurea*, *E. angustifolia*, and *E. pallida*, are commonly used in current botanical preparations (medicinal species). The use of these medicinal species originated from Native American peoples who utilized *Echinacea* roots, aboveground parts, or a combination of both as treatments for different ailments ranging from toothache to rheumatism and as an antidote for poisons and venoms (*2*).

Four classes of active compounds have been identified within *Echinacea*, yielding different chemical profiles among its nine species (*3*). It has been hypothesized that alkamides, caffeic acid derivatives, polysaccharides, and glycoproteins are the classes of compounds responsible for the bioactivity of *Echinacea* (*4*). *Echinacea purpurea* contains alkamides, caffeic acid esters (in particular cichoric acid), polysaccharides, and polyacetylenes, whereas in *Echinacea pallida* alkamides are mostly absent and the most abundanat caffeic acid ester is echinacoside (*5*). Furthermore, levels of constituents vary during growth and across development (*3*, *5*).

The chemical diversity these plants exhibit has made it difficult for researchers to determine if *Echinacea* can be

* Author to whom correspondence should be addressed [telephone (515) 294-9873; e-mail dbirt@iastate.edu].
† Center for Research on Dietary Botanical Supplements, Iowa State University and the University of Iowa.
‡ Interdepartmental Genetics Graduate Program, Iowa State University.
§ Department of Food Science and Human Nutrition, Iowa State University.
# Department of Genetics, Development, and Cell Biology, Iowa State University.
|| Department of Chemistry, Iowa State University.
⊥ Department of Statistics, Iowa State University.
⊗ Daejin University.
△ Sunchon National University.
× Departments of Horticulture and Agronomy, Iowa State University.
◇ Plant Introduction Research Unit, Agricultural Research Service, U.S. Department of Agriculture.

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effective in treating colds and other respiratory infections (*6*). Inconsistent results have been obtained from several placebo-controlled studies designed to determine whether *Echinacea* preparations were effective in the prevention of the common cold and other upper respiratory infections. These conflicting results were perhaps due to the use of different species and plant parts, different preparations and doses, inconsistent times of treatment initiation, and different virus types and doses (*5*, *6*). Some studies revealed a shorter duration of cold symptoms after *Echinacea* treatment in comparison to placebo (*7*, *8*), whereas others showed no significant differences between treatment and placebo groups (*9*, *10*). Although the controversy regarding efficacy continues, studies are accumulating that indicate *Echinacea* may have antiviral, antioxidant, and anti-inflammatory properties, making it a very promising medicinal botanical (*4*, *11*, *12*).

Alkamides, also known as alkylamides, are a major group of lipophilic, bioactive phytochemicals found abundantly in certain species of *Echinacea*. Evidence indicates that alkamides possess anti-inflammatory properties because they have been shown to significantly reduce nitric oxide production in lipopolysaccharide (LPS) stimulated RAW264.7 macrophages (*12*). Thus, much attention is being directed toward alkamides to better understand their potential use as anti-inflammatory agents as well as how they interact with other constituents found in *Echinacea* (*12*).

Prostaglandin E_2 (PGE_2) is a critical inflammatory mediator that is produced through the arachidonic acid cascade. Two cyclooxygenase isoforms, COX-1 and COX-2, catalyze the reaction converting arachidonic acid, released by phospholipase A, to PGE_2. LPS is a common endotoxin used to stimulate macrophage cells to produce PGE_2, mimicking an inflammatory response in vitro (*13*). The use of RAW264.7 mouse macrophage cells has been established as a reliable cell model for purposes of identifying anti-inflammatory activity (*12*).

The purpose of our study was to compare the bioactivities of species of *Echinacea* and assess levels of variability on the basis of repeat extractions and different harvest years. It was also important to determine whether specific alkamides play a clear role in the anti-inflammatory properties of six *Echinacea* species. To this end, we have conducted (to our knowledge) the first large-scale screen of nine synthesized alkamides found in *Echinacea*, three synthesized analogues, two of Bauer alkamide 12 (*14*) and one of Chen alkamide 2, and one synthesized isomer of Chen alkamide 1 (*12*) for their ability to reduce LPS-stimulated PGE_2 production.

MATERIALS AND METHODS

Plant Material and Extraction. Plant samples were provided by Frontier Natural Products Co-op (FNPC, Norway, IA) and the North Central Regional Plant Introduction Station (NCRPIS, Ames, IA) of the Agricultural Research Service of the U.S. Department of Agriculture. The FNPC supplied *E. purpurea*, which had been harvested during its budding stage in Bulgaria in 1999, where it was air-dried and, in 2001, shipped to FNPC. Roots of plants representing the following 10 *Echinacea* accessions were obtained from the NCRPIS, where they were harvested in October 2003, November 2004, and November 2005: cultivated populations of *E. purpurea* (Ames 28189), *E. angustifolia* (Ames 28187), and *E. pallida* (Ames 28188), all originally acquired from Johnny's Selected Seeds (Winslow, ME), and wild populations of *E. purpurea* (PI 631307 and PI 633665), *E. angustifolia* (PI 631285), *E. pallida* (PI 631293), *E. simulata* (PI 631251), *E. sanguinea* (PI 633672), and *E. tennesseensis* (PI 631250). Information about the specific provenance of all accessions obtained from the NCRPIS is available on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html. Roots were harvested, and the plant material was prepared for storage by drying for 8 days at 38 °C in a forced-air dryer with constant humidity. The dried material was ground with a 40-mesh screen and stored at −20 °C until extraction. Extractions were made by using 6 g of dried *Echinacea* root per population.

Extracts were prepared by one of two methods, either the Soxhlet method (6 h) or room temperature shaking (24 h). Solvents ranging in hydrophobicity were used for extraction, consisting of ethanol (70, 95, or 100%), water, chloroform (100%), hexane (100%), or sequential extractions. Sequential extractions were performed by extraction first with chloroform (70, 95, or 100%) or hexane (70%), removal of the solvent, and then evaporation. The residue plant material was re-extracted with ethanol. FNPC plant material was extracted with either 100% ethanol using the Soxhlet method (heating solvent to its boiling point) or shaking with 70% ethanol at room temperature. The Soxhlet method was determined to yield optimal material and was therefore used to extract NCRPIS *Echinacea* material with 95% ethanol. Upon complete drying of the extract by evaporation, the residue was redissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at the highest concentration that was soluble. Extracts were stored at −30 °C in the dark and used as stock solutions.

Endotoxin. *Echinacea* extracts from NCRPIS were all screened for endotoxin by using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to the manufacturer's specifications for a microplate assay. After accounting for the dilutions used in the PGE_2 assay, the range of endotoxin levels presented to RAW264.7 macrophage cells varied from undetectable to 0.0082 EU/mL. At these levels the endotoxin found was well below the required amount needed (>5 EU/mL) to induce the production of PGE_2 in RAW264.7 cells (*15*).

Cell Culture. RAW264.7 mouse monocyte/macrophage cells were obtained from American Type Culture Collection (catalog no. TIB-71, Manassas, VA) and cultured as described by Hammer et al. (*15*). NIH/3T3 mouse fibroblast cells, SW480 human colon cancer cells, and HaCaT human skin cancer cells were cultured according to procedures described by Schmitt et al. (*16*).

Alkamide Synthesis. Chemical synthesis of Bauer (*14*) and Chen alkamides (*12*) were conducted according to the procedures outlined by Wu et al. (*17*), Kraus and Bae (*18*), and the thesis of Jaehoon Bae (Iowa State University, 2006) (*19*). The synthesized alkamides allowed for the comparison of activity of purified alkamide constituents, both those found in *Echinacea* and derivatives of those alkamides.

Measurement of Prostaglandin E_2. *Echinacea* extract and alkamide treatments of RAW264.7 cells and the PGE_2 enzyme immunoassay (EIA) used to detect the amount of PGE_2 (GE Biosciences, Piscataway, NJ) were previously described by Hammer et al. (*15*). Preparations of *E. purpurea* extracts from FNPC were extracted with several solvents including Soxhlet EtOH, room temperature (RT) EtOH, Soxhlet chloroform, and Soxhlet hexane, as well as sequential Soxhlet extracts with chloroform/EtOH and hexane/EtOH. Initial screens for PGE_2 production comparing different solvents resulted in significant increases in PGE_2 levels with all solvents except Soxhlet EtOH, RT EtOH, and Soxhlet chloroform in the absence of LPS. These results as well as the common use of ethanol in herbal supplements guided our laboratory to use Soxhlet EtOH extract preparations in our assays. Also, Soxhlet EtOH extracts performed optimally in our assays, compared to water extracts that generally had higher endotoxin contamination (data not shown). Work conducted by Bauer et al. (*14*) supports the use of ethanol extractions of *Echinacea*, which allow for the enrichment of lipophilic compounds including the ethanol-soluble alkamides (*20*). Baicalein (5,6,7-trihydroxyflavone), found in the Chinese medicinal herb *Scutellaria baicalensis*, and quercetin (3,5,7,3′4′-pentahydroxy flavon), present in the aboveground parts of *E. purpurea* (*21*), are flavonoids that are known to exert anti-inflammatory as well as antioxidant effects and were used as positive controls for the PGE_2 assay due to these properties (baicalein was synthesized by Iowa State University, and quercetin was purchased from Sigma-Aldrich).

Cytotoxicity. Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) was used to analyze cytotoxicity following a modified version of Schmitt et al.'s protocol (*16*). RAW264.7 cells were plated into 48-well plates at a density of 0.5×10^5cells/well and incubated at 37 °C for 24 h before treatment.

Treatments were prepared by diluting the 6 g of *Echinacea* extract or alkamide in DMSO and then further diluting with media to the concentrations specified under Results. The stock extracts diluted to 1% of the total treatment concentration (0.1% if shown to be cytotoxic at 1%) or synthesized alkamide diluted to 0.1% of the total treatment concentration were randomly assigned to plate wells and incubated for 24 h (8 h if shown to be cytotoxic at 24 h) along with pure media and DMSO as solvent controls. Ursolic acid, a triterpenoid known for its cytotoxic activities (*22*), was used as a positive control at concentrations of 10, 30, and 50 μM, yielding significant cytotoxicity ($p < 0.0001$) at the two higher concentrations. Following the 24 h incubation period, treatment solutions were removed, and fresh media and Celltiter96 dye were added for 195 min, which was found to be the optimal incubation time for this study. The metabolized dye solutions were then transferred to 96-well plates for absorbance measurement at 562 nm. The number of viable cells for each treatment was compared to the media + DMSO control, and the percent of control survival was determined for each extract or alkamide.

High-Performance Liquid Chromatography Analysis. The HPLC method was described in previously published work (*17*, *23*). Briefly, into 320 μL of *Echinacea* extracts were added 40 μL (1 mg/mL) of *N*-isobutylundeca-2-ene-8,10-diynamide ($C_{15}H_{21}O_2$) and 40 μL (1 mg/mL) of 3,5-dimethoxy-4-hydroxycinnamic acid ($C_{11}H_{12}O_5$) as internal standards for quantification of lipophilic metabolites and hydrophilic metabolites, respectively. Fifteen microliters of each extract was injected into a HPLC. The instrumentation and solvent system for HPLC separation were the same as those previously published (*23*). For compound identification, Bauer alkamides 8/9, cichoric acid, echinacoside, caftaric acid, and cynarin were purchased from Phytolab (Vestenbergsgreuth, Germany); chlorogenic acid was purchased from Sigma-Aldrich; and Bauer alkamides 2 and 10−14 and ketones 20−22 were synthesized (*17*, *18*). Peaks were identified according to retention time and mass spectra obtained from LC-MS and/or GC-MS. In the absence of standards, Bauer alkamides 1, 3, 4, 5, 7, 15, 16, and 17 and ketone 24 were identified by HPLC fractionation coupled with GC-MS analysis. Specifically, eluted HPLC fractions were collected and subsequently subjected to GC-MS analysis; six replicate runs were carried out, and appropriate peaks were pooled to ensure sufficient yield of each alkamide. Compounds contained in each pooled fraction were identified by comparing their recorded mass spectra and online UV spectra with those from published literature (*24*). Compounds were quantified on the basis of internal standards. The percent repeatability and limits of detection for HPLC quantification of Bauer alkamides 2, 8/9, 10, 11, 12, 13, and 14, ketones 20, 21, and 22, caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid with reference standards range between 1.64 and 2.86% and between 0.02 μg/mL and 0.16 μg/mL, respectively. The repeatability was determined by analyzing repeated injections of the standard solution ($n = 6$). The standard deviation values of the repeatability are <3%, illustrating the precision of the HPLC method.

Statistical Analysis. For both PGE_2 data and cytotoxicty data, in separate analyses, the results were log transformed and normalized to the media + DMSO control allowing for the combination of treatments on different plates. Cytotoxicity was analyzed by using a mixed model, in which the plate was considered a random effect. A two-way ANOVA was used followed by a Dunnett multiple-comparison test (*25*) for PGE_2 and cytotoxicity assays. Data are represented as percent of media + DMSO control set to 100%. Statistical significance was defined as $p < 0.05$, and 95% confidence intervals were used. PGE_2 data from alkamide treatments were analyzed the same as the *Echinacea* extracts, except data are expressed as mean percent reduction as compared to media + DMSO control set at 0%. The statistical program used for all analyses was SAS 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

Extracts from *Echinacea* Species Inhibit PGE_2 Production. To assess the anti-inflammatory properties of six *Echinacea* species, Soxhlet EtOH extracts were initially screened in RAW264.7 cells at their highest concentration (ranging in final concentrations from 21 to 53 μg/mL) for their ability to reduce PGE_2 levels after stimulation with LPS (**Figure 1**). *E. angustifolia* and *E. pallida* extracts from NCRPIS and Johnny's Selected Seeds significantly inhibited PGE_2 levels at concentrations above 21 μg/mL. Neither *E. purpurea* accession screened in **Figure 1** significantly inhibited PGE_2 (28 μg/mL of extract from NCRPIS and 24 μg/mL of extract from Johnny's Selected Seeds). Treatments analyzed without the addition of LPS reduced PGE_2 levels in *E. purpurea* and *E. angustifolia* compared to media + DMSO control. Baicalein and quercetin were included as positive controls for every PGE_2 experiment. After initial screening, the extracts were diluted to 15 μg/mL in DMSO for activity comparisons across species. There was no significant difference among harvest year, accession, or Soxhlet EtOH extract preparation for *E. purpurea* (six extracts), *E. angustifolia* (four extracts), *E. pallida* (four extracts), *E. sanguinea* (two extracts), *E. simulata* (two extracts), or *E. tennesseensis* (two extracts), which allowed data to be pooled. Of the three medicinal species, *E. angustifolia* and *E. pallida* significantly inhibited PGE_2 levels ($p < 0.05$) (**Figure 2**). Three nonmedicinal species were also screened for anti-inflammatory activity. *E. sanguinea* and *E. simulata* significantly reduced PGE_2 production ($p < 0.001$ and $p < 0.05$, respectively). Of the six species being compared in this study, *E. purpurea* and *E. tennesseensis* showed the least activity in this assay. Of the four active species that reduced PGE_2 production, none was significantly more active than the other when confidence intervals across species were compared.

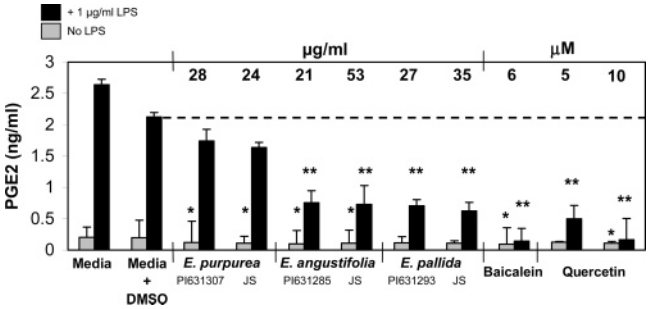


Figure 1. Inhibition of PGE_2 production by Soxhlet ethanol extracts of medicinal species of *Echinacea* treated at their highest concentration (6 g of extract diluted initially in DMSO and then prepared as 0.1% of the media). Each bar represents three replicates ± standard deviation. Dry material was obtained from the 2005 harvests grown by NCRPIS. Johnny's Selected Seeds accessions are denoted JS on the graph, and PI is indicative of accessions from NCRPIS. ∗, $p < 0.05$, and ∗∗, $p < 0.001$, for comparison of extract to control. Media + DMSO and media + DMSO + LPS treatments are represented by gray and black bars, respectively.

Screening for Cytotoxicity of Extracts of *Echinacea* Species. To determine whether any of the NCRPIS Soxhlet EtOH extracts had the ability to arrest metabolic activity in RAW264.7 macrophage cells and to provide further evidence that the observed PGE_2 reduction with treatment of *Echinacea* extracts was not due to cytotoxicity, a parallel study using the Celltiter96 Aqueous One Solution Cell Proliferation Assay was conducted. **Table 1** displays an initial screening of each extract at a 1% concentration of extract diluted in media for a 24 h incubation. This initial screening used 10-fold higher concentrations than those used in the PGE_2 screens. All extracts showed significant cytotoxicity ($p < 0.0001$), with 25−72% survival at the 1% concentration (ranging from 240 to 1102 μg/mL of extract) and 24 h of incubation.

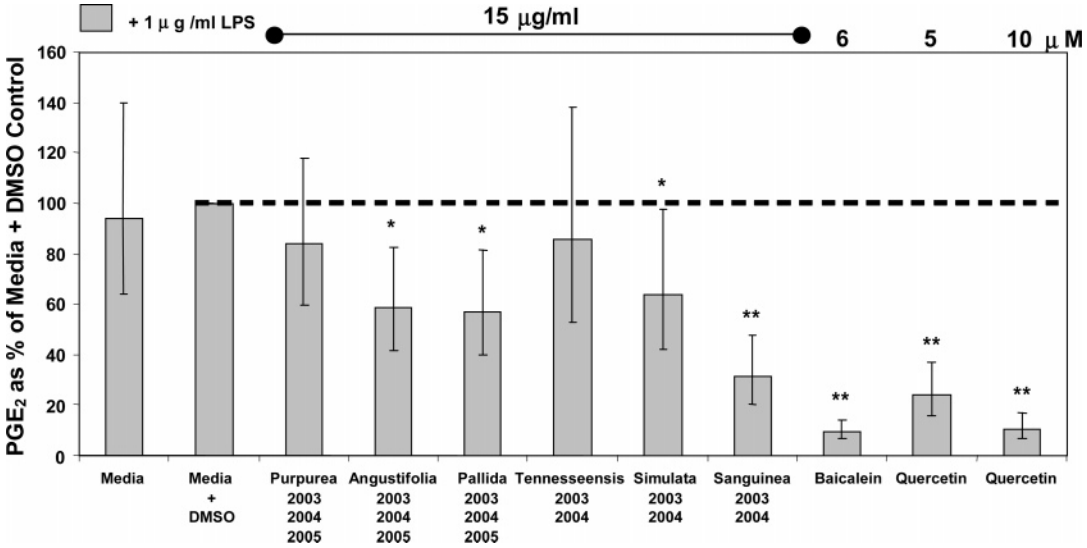


Figure 2. Inhibition of PGE_2 production by extracts of three medicinal and three nonmedicinal species of *Echinacea* (diluted to 15 μg/mL of extract in DMSO) obtained from NCRPIS with different harvest dates and accession numbers. Each species mean is represented by a bar (two to six replicates each), and variability is represented as 95% confidence intervals of the mean. Treatments analyzed without the addition of LPS did not affect PGE_2 levels with values for PGE_2 as percent of media + DMSO control <20% (data not shown). One *E. angustifolia* extract, from the 2004 harvest, treated without LPS, was excluded from the analysis on the basis of the optical density data point being outside the standard curve. $*$, $p < 0.05$, and $**$, $p < 0.001$, for comparison of extract to control (media + DMSO + LPS).

Table 1. Cytotoxicity Screening of Soxhlet EtOH Extracts from Six *Echinacea* Species[a]

species	harvest year	accession	1% concn (μg/mL)	% survival (95% CI), *p* value	0.1% concn (μg/mL)	% survival (95% CI), *p* value
E. angustifolia	2005	JS	535	71 (53, 97), **0.0166**	54	92 (74, 116), 0.9792
E. purpurea	2003	PI 633665	518	65 (48, 88), **0.0004**	52	104 (79, 139), 1.0000
E. purpurea	2005	JS	240	72 (53, 97), **0.0181**	24	105 (78, 139), 1.0000
E. pallida	2003	PI 631293	579	68 (50, 92), **0.0021**	58	75 (56, 99), **0.0365**
E. pallida	2005	JS	359	32 (26, 40), **<0.0001**	36	133 (100, 177), 0.0457
E. tennesseensis	2003	PI 631250	950	66 (53, 83), **<0.0001**	95	112 (84, 149), 0.9431
E. simulata	2003	PI 631251	1101	25 (20, 32), **<0.0001**	110	102 (77, 136), 1.0000
E. simulata	2004	PI 631251	1102	25 (20, 30), **<0.0001**	110	110 (83, 146), 0.9817
E. sanguinea	2003	PI 633672	834	36 (28, 45), **<0.0001**	83	93 (74, 116), 0.9874
E. sanguinea	2004	PI 633672	672	34 (27, 43), **<0.0001**	67	96 (73, 128), 1.0000

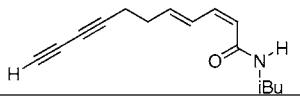
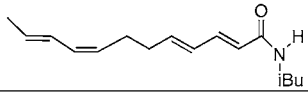
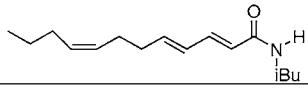
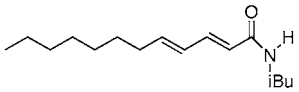
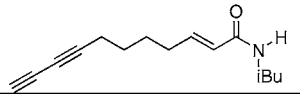
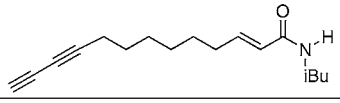
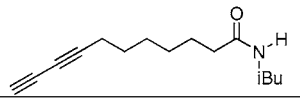
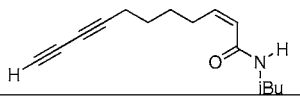
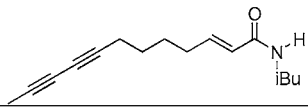
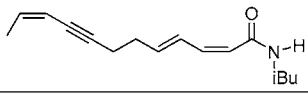
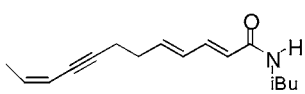
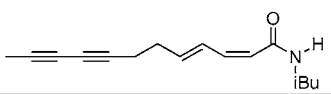
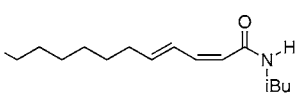
[a] Cytotoxicity (percent of control compared to vehicle control-treated RAW264.7 cells) of *Echinacea* extracts screened via the Celltiter96 Aqueous One Solution Cytotoxicity assay ($n = 3$–4). PI numbers denote NCRIPS accessions, whereas JS denotes accessions from Johnny's Selected Seeds. All extract stocks were prepared from 6 g of dried root plant material by Soxhlet 95% EtOH extraction, were diluted in DMSO and included as 1% of the cell culture medium or, if significantly cytotoxic at 1%, diluted to 0.1%. The treatment concentration listed for each extract (μg/mL) is the amount of extract residue used in the assay after extraction and dilution in DMSO. *p* value for comparison of extract treatment to control (media + DMSO). Boldface *p* values represent statistical significance with $p < 0.05$.

All extracts cytotoxic at the 1% concentration were screened again at the 0.1% concentration of extract diluted in media (concentrations ranging from 24 to 110 μg/mL of extract) after a 24 h incubation. Results in **Table 1** show that only one extract of *E. pallida* from the 2003 harvest (PI 631293) remained significantly cytotoxic at the 0.1% dilution after a 24 h incubation, but when this same extract was screened for cytotoxicity at the same dilution after an 8 h incubation, equivalent to the incubation period in the PGE_2 assay, no significant cytotoxicity remained ($p = 0.9968$). The screens conducted at 0.1% concentration matched concentrations used in PGE_2 screens.

Cytotoxicity Screen of FNPC *E. purpurea* Extracts. Viability of NIH/3T3 mouse fibroblast cells, SW480 human colon cancer cells, and HaCaT human skin cancer cells treated with FNPC *E. purpurea* extracts was assessed by using the Celltiter96 Aqueous One Solution Cell Proliferation Assay. Cytotoxicity was not observed in any of the FNPC *E. purpurea* extracts at a concentration of 10 μg/mL. Significant cytotoxicity was observed only at high concentrations ($\geq$95 μg/mL of extract), with the exception of one extract showing significant cytotoxicity ($p < 0.01$) at 39 μg/mL [(RT) 70% hexane 24 h, SW480 cells]. This extract protocol was not used in further studies. Results from these data indicate that all other extracts prepared from FNPC *E. purpurea* showed little or no cytotoxicity in the three different cell lines at concentrations of <100 μg/mL.

Synthesized Alkamides from *Echinacea* Species Inhibit PGE_2 Production. To further probe the inhibition of PGE_2 production in RAW264.7 cells observed with treatments of *Echinacea* extracts, another PGE_2 screening was conducted on chemically synthesized alkamides, one of *Echinacea*'s major classes of bioactive constituents. Data from **Table 2** show that all of the synthesized alkamides screened significantly inhibited the production of PGE_2 ($p < 0.001$) at 50 μM. At 25 μM, Bauer alkamide 8, Bauer alkamide 12A analogue, Bauer alkamide 14, Chen alkamide 2, and Chen alkamide 2 analogue significantly reduced PGE_2 levels ($p < 0.05$). Only Bauer alkamide 14 significantly inhibited PGE_2 production at 10 μM ($p < 0.05$). A subsequent PGE_2 EIA experiment was conducted to attempt to determine if PGE_2 could inhibit at concentrations of <10

Table 2. Inhibition of PGE_2 Production by Alkamides

Alkamide Name	Alkamide Structure	% Reduction (95% CI) p-value * 50 μM	25 μM	10 μM
Bauer alkamide 2 Undeca-2*Z*, 4*E*-diene-8,10-diynoic acid isobutylamide		55 (30, 71) **0.0023**	32 (-6, 56) 0.1371	22 (-21, 50) 0.3463
Bauer alkamide 8 Dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide		87 (76, 93) **<0.0001**	75 (53, 87) **<0.0001**	46 (-0.8, 71) 0.0730
Bauer alkamide 10 Dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide		90 (81, 95) **<0.0001**	46 (-2, 71) 0.0781	42 (-8, 69) 0.1110
Bauer alkamide 11 Dodeca-2*E*, 4*E*-dienoic acid isobutylamide		71 (46, 84) **0.0004**	24 (-42, 59) 0.4281	45 (-3, 71) 0.0816
Bauer alkamide 12 Undeca-2*E*-ene-8, 10-diynoic acid isobutylamide		59 (41, 72) **<0.0001**	35 (-2, 58) 0.1029	39 (5, 61) 0.0580
Bauer alkamide 12A analogue + Trideca-10, 12-diynoic acid isobutylamide		90 (82, 95) **<0.0001**	73 (50, 86) **0.0002**	39 (-13, 68) 0.1448
Bauer alkamide 12B analogue + 2, 3dihydro Bauer amide 12		56 (19, 77) **0.0163**	35 (-22, 65) 0.2110	29 (-32, 62) 0.3110
Bauer alkamide 13 Undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide		74 (51, 86) **0.0001**	48 (3, 72) 0.0555	30 (-32, 62) 0.3089
Bauer alkamide 14 Dodeca-2*E*-ene-8, 10-diynoic acid isobutylamide		74 (59, 83) **<0.0001**	71 (54, 81) **<0.0001**	45 (14, 64) **0.0240**
****Chen alkamide 1** Dodeca-2*Z*, 4*E*, 10*Z*-trien-8-ynoic acid isobutylamide		74 (51, 86) **0.0001**	32 (-28, 63) 0.2710	29 (-32, 62) 0.3129
Chen alkamide 1 isomer + Dodeca-2*E*, 4*E*, 10*Z*-trien-8-ynoic acid isobutylamide		65 (34, 81) **0.0026**	31 (-29, 63) 0.2751	27 (-37, 61) 0.3675
****Chen alkamide 2** Dodeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide		84 (70, 92) **<0.0001**	54 (15, 76) **0.0234**	24 (-42, 59) 0.4248
Chen alkamide 2 analogue + Dodeca-2*Z*, 4*E*-dienoic acid isobutylamide		89 (80, 94) **<0.0001**	78 (59, 88) **<0.0001**	40 (-11, 68) 0.1332

**p* value for comparison of alkamide treatment to control (media + DMSO + LPS). Boldface *p* values represent statistical significance as defined as a *p* value of <0.05. Media + DMSO + LPS control set to 0% reduction. There was no difference between extracts in medium alone having an overall percent reduction (95% CI) of 2 (−27, 24) and media + DMSO controls. Baicalein (6 μM) and quercetin (10 μM) were used as positive controls having overall percent reductions (95% CI) of 72 (62, 79) and 88 (85, 91), respectively. All samples in the table are treated with 1 μg/mL LPS. Alkamide treatments did not affect PGE_2 levels without LPS treatment (data not shown). Bauer alkamides are found in Bauer et al. (*14*) and **Chen alkamides in Chen et al. (*12*). Isomers and analogues of naturally occurring alkamides are indicated with the + symbol and have not been detected to date from *Echinacea* species extracts in our laboratories.

Table 3. HPLC Analysis of Soxhlet EtOH Extracts of *Echinacea* Species (15 μg/mL)[a]

Species	*E. angustifolia*		*E. pallida*			*E. simulata*		*E. sanguinea*		*E. purpurea*					*E. tennesseensis*	
Accession (PI63)	1285	1285	1293	1293	1293	1251	1251	3672	3672	3665	3665	1307	1307	1307	1250	1250
Harvest Date	2003	2003	2003	2003	2004	2003	2004	2003	2004	2003	2004	2003	2003	2004	2003	2004
Bauer Alkamide 1	0.050	0.051	0.024	0.008	0.006	-	0.003	0.071	0.165	0.254	0.265	0.595	0.296	0.153	0.030	0.029
Bauer Alkamide 2	0.124	0.040	0.132	0.014	0.023	0.051	0.044	0.048	0.082	0.253	0.265	0.590	0.848	0.600	-	-
Bauer Alkamide 3	0.155	0.101	0.069	-	0.012	0.008	0.009	0.160	0.403	0.327	0.395	1.280	0.250	0.375	-	-
Bauer Alkamide 4	-	-	-	-	-	-	-	-	-	0.085	0.079	0.071	0.039	0.048	-	-
Bauer Alkamide 5	0.082	0.092	0.019	0.004	0.004	-	-	0.014	0.034	0.083	0.088	0.205	0.089	0.065	-	-
Bauer Alkamide 7	0.032	0.009	0.018	0.005	0.019	-	-	0.046	0.105	0.173	0.199	0.250	0.065	0.131	-	-
Bauer Alkamide 8	0.331	0.206	-	-	-	-	-	0.223	0.610	0.263	0.269	0.902	0.770	0.458	0.011	0.011
Bauer Alkamide 9	2.800	1.642	-	-	-	-	-	0.458	1.151	0.403	0.420	1.612	1.687	0.866	0.027	0.057
Bauer Alkamide 10	0.166	0.137	-	-	-	-	-	0.031	0.075	0.064	0.064	0.035	0.016	0.013	0.009	-
Bauer Alkamide 11	0.430	0.403	-	-	-	-	-	0.018	0.044	0.047	0.050	0.052	0.020	0.010	0.006	0.013
Bauer Alkamide 12	0.395	0.244	-	-	-	-	-	0.292	0.549	-	-	-	-	-	0.307	0.336
Bauer Alkamide 13	0.386	0.140	-	-	-	-	-	0.276	0.500	-	-	-	-	-	0.622	0.677
Bauer Alkamide 14	0.156	0.086	-	-	-	-	-	0.430	0.741	-	-	-	-	-	0.087	0.095
Bauer Alkamide 15	-	-	0.009	0.005	0.011	-	-	-	-	0.022	0.023	0.009	0.017	0.010	-	-
Bauer Alkamide 16	-	-	-	-	-	-	-	0.027	-	-	-	-	-	-	0.096	0.100
Bauer Alkamide 17	0.044	0.016	-	-	-	-	-	0.169	0.327	-	-	-	-	-	0.021	0.023
Chen Alkamide 2	0.026	0.009	0.037	0.018	0.013	0.023	0.024	0.059	0.148	0.075	0.087	0.190	0.114	0.129	-	-
Ketone 20	-	-	0.099	0.057	0.300	0.069	0.120	-	0.026	-	-	-	-	-	-	-
Ketone 21	-	-	0.030	0.021	0.047	0.014	0.015	-	-	-	-	-	-	-	-	-
Ketone 22	-	-	0.653	0.432	0.153	0.148	0.137	-	-	-	-	-	-	-	-	-
Ketone 24	0.032	0.018	0.810	0.360	0.131	0.079	0.076	0.052	0.095	-	-	-	-	-	-	-
Caftaric acid	-	-	0.003	0.008	0.004	0.009	0.004	0.007	0.002	0.034	0.011	0.022	0.045	0.026	-	-
Chlorogenic acid	0.010	0.008	0.004	0.004	0.011	0.003	0.001	0.073	0.037	0.026	0.007	0.003	0.005	0.004	0.021	0.009
Cynarin	0.072	0.034	-	-	-	-	-	0.008	0.001	0.017	0.002	0.007	0.017	0.009	0.078	0.027
Echinacoside	0.040	0.037	0.104	0.111	0.075	0.079	0.040	0.015	0.011	-	-	-	-	-	-	-
Cichoric acid	-	-	-	-	-	-	-	-	-	0.289	0.075	0.078	0.127	0.089	-	-

[a] The units for values in the table are all μM. The HPLC analysis identifies three main classes of constituents found in the species of *Echinacea* extracts (15 μg/mL of extract) obtained from NCRPIS, namely, alkamides, ketones, and caffeic acid derivatives. Shaded cells identify compounds for which concentrations exceed 0.1 μM. Due to the repeatability of our HPLC method, described under Materials and Methods, constituents are represented by an $N = 1$; therefore, no statistical analysis was performed on these data. Dashes represent values below instrumental detection limits. Structures and nomenclature of Bauer alkamides 2, 8, and 10–14 presented in the above table were previously shown in **Table 2**, and other alkamides and ketones can be found in Bauer et al. (*14*). Structures of caffeic acid derivatives have previously been reported.

μM. After storage of Bauer alkamide 14 at −24 °C for 1 year, HPLC analysis indicated that it had degraded to 31% of the original concentration. The concentrations screened for PGE_2 production were 3.1 and 0.230 μM, and Bauer alkamide 14 was unable to significantly inhibit PGE_2 at these concentrations (data not shown).

Screening for Cytotoxicity of Synthesized Alkamides. Parallel cytotoxicity screenings were carried out for the alkamides by using the Celltiter96 Aqueous One Solution Cell Proliferation Assay in RAW264.7 mouse macrophage cells. The alkamides were all screened at 50 μM for a 24 h incubation. None of the 13 alkamides demonstrated any significant cytotoxicity (85−113% survival), indicating that cell death was not a factor in the data obtained from the PGE_2 screening (data not shown).

HPLC Analysis of *Echinacea* Extracts at 15 μg/mL. HPLC was performed with 15 μg/mL of *Echinacea* extracts from NCRPIS to identify and analyze innate concentrations of known constituents (**Table 3**). *E. angustifolia*, *E. sanguinea*, *E. purpurea*, and *E. tennesseensis* contained greater quantities of Bauer alkamides than of ketones or caffeic acid derivatives. Bauer alkamides 3 and 8−13 were present in *E. angustifolia* at concentrations of >0.1 μM, with Bauer alkamide 9 reaching concentrations of 2.8 and 1.6 μM in the two extracts analyzed. All accessions of *E. sanguinea* also contained Bauer alkamides 3, 8, 9, 12−14, and 17 at concentrations ranging from 0.16 to 1.15 μM. Two different accessions of *E. purpurea* (PI 63665 and PI 631307) were analyzed by HPLC and were found to both contain similar alkamides, including Bauer alkamides 1−3 and 7−9 (>0.1 μM). Cichoric acid was present in one extract of each accession from the 2003 harvest of *E. purpurea*. *E. tennesseensis* (2003 and 2004 harvests) contained Bauer alkamide 12 at concentrations of >0.3 μM and Bauer alkamide 13 at concentrations of >0.6 μM. Two species, *E. pallida* and *E. simulata*, were determined to possess greater amounts of ketones than of alkamides. *E. pallida* had concentrations exceeding 0.1 μM for ketones 22 and 24. Ketone 22 was also present in *E. simulata* extracts (>0.1 μM of ketone). Chen alkamide 1, Chen alkamide 1 isomer, Chen alkamide 2 analogue, Bauer alkamide 12A analogue, and Bauer alkamide 12B analogue from **Table 2** were not detected in this analysis, but may be present in concentrations below the detection limits of our instrument. Some alkamides, ketones, and caffeic acid derivatives were present in only certain extracts within the species and are identified in **Table 3**.

DISCUSSION

The results presented in this study demonstrate the inhibition of PGE_2 by several *Echinacea* species, which may be one process contributing to the reported anti-inflammatory capabilities of these herbs (*12*). All Soxhlet EtOH extracts from the six species screened, when tested at their highest concentration, reduced PGE_2 levels, except *E. purpurea*. To compare inhibition potential among these species, extracts were diluted to 15 μg/mL in DMSO, with *E. angustifolia*, *E. pallida*, *E. sanguinea*, and *E. simulata* significantly inhibiting the production of PGE_2 in LPS-induced RAW264.7 mouse macrophage cells. *E. purpurea* extracts (15 μg/mL) did not significantly inhibit PGE_2, which was notable because many *Echinacea* supplements

contain *E. purpurea* as a major ingredient (*21*). Another interesting finding was that variability between species was greater than that observed between repeat extractions or harvest years. Results obtained from screening *Echinacea* extracts in RAW264.7 cells for cytotoxicity coincide with observations from NIH/3T3 mouse fibroblast cells, SW480 human colon cancer cells, and HaCaT human skin cancer cells, which showed these extracts to be cytotoxic only at high concentrations (>240 μg/mL of extract).

Alkamides have become a major focus for researchers studying *Echinacea*, due to their abundance in both aboveground and underground parts of the plant in most species. Studies link this class of compounds to a vast repertoire of immunomodulatory activities, including antiviral, antimicrobial, antibacterial, and antioxidant as well as anti-inflammatory properties (*26*). Alkamides may be best known through recent studies indicating their ability to modulate the immune system, potentially, by binding to the cannabinoid 2 receptor (CB2) (*27*). This receptor has been shown to be expressed in many types of inflammatory and immune-competent cells, and it has been suggested that the CB2 receptor may play a part in inflammatory reactions (*28*). Alkamides have been shown, through the use of CB1 and CB2 antagonists and signal transduction pathway inhibitors, to up-regulate TNF-α mRNA and increase cAMP, p38/MAPK, and JNK signaling, as well as activate NF-kB through the CB2 receptor in human monocyte/macrophage cells (*29*).

Our results provide further support to previous studies indicating that alkamides are key constituents found in *Echinacea* that possess anti-inflammatory properties. Our experiments indicated that alkamides are consistent inhibitors of PGE_2 production at a concentration of 50 μM, with selected alkamides having the capability to significantly inhibit PGE_2 levels at concentrations of 25 μM or even 10 μM. Although many alkamides have been identified, it is possible that undescribed isomers as well as analogues of many of the known alkamides may exist naturally in some of the *Echinacea* species, and on the basis of our results, some of these may also be inhibitors of inflammatory mediators. Cytotoxicity did not appear to contribute to reduced PGE_2 production by extracts or alkamides, suggesting that the observed inhibition was a true inhibition of PGE_2 and not an artifact due to cell death. The data presented strengthen the research indicating that alkamides present in *Echinacea* contribute to immunomodulatory properties dealing with regulation of inflammation (*27*).

Questions as to how alkamides act together and in concert with other constituents arise from our HPLC analysis. Our data identify alkamides present in crude *Echinacea* extracts at concentrations below 2.8 μM, which is well under the 10 μM concentration observed for inhibitory activity of PGE_2 seen with chemically synthesized Bauer alkamide 14. Regardless, several of the crude extracts of *Echinacea*, containing a variety of alkamides, ketones, and caffeic acid derivatives at low concentrations, were able to significantly inhibit PGE_2 production. For example, *E. sanguinea* extracts at 15 μg/mL, containing Bauer alkamides 8 and 12−14, at higher concentrations than other constituents present, showed the greatest inhibition of PGE_2 among the species we evaluated, with a percent reduction of 59%, whereas none of these alkamides would be able to inhibit PGE_2 production alone at such low concentrations. Therefore, we hypothesize that the noted inhibition of PGE_2 and, by extension, the anti-inflammatory properties found in *Echinacea* extracts were not simply due to one constituent, but several acting in a synergistic or additive manner. Synergism has been reported previously between alkamides and caffeic acid derivatives and their ability to inhibit the oxidation of low-density lipoproteins, as an indicator of antioxidant activity (*4*). Our HPLC analysis revealed no clear pattern of identifiable constituents that led to the observed inhibition of PGE_2 by extracts from *E. angustifolia*, *E. pallida*, *E. simulata*, or *E. sanguinea*, indicating that more research is needed to understand the complex nature of interacting constituents within each species and to determine mechanisms behind the identified PGE_2 inhibition. A possible explanation for the resemblance of constituents found in *E. pallida* and *E. simulata* may be related to the hypothesis that *E. simulata* is one of the diploid progenitors of the tetraploid species, *E. pallida* (*30*). A PGE_2 screening of synthesized ketones and caffeic acid derivatives individually may lead to a better understanding of *Echinacea*'s anti-inflammatory capabilities.

A study performed by Chen et al. (*12*) indicated that alkamides had anti-inflammatory activity as measured by inhibition of nitric oxide (NO) production in LPS-stimulated RAW264.7 cells. NO is a pro-inflammatory mediator that was significantly reduced by total alkamide (a mixture consisting of several alkamides) ranging from 1.6 to 30 μg/mL. Chen et al. (*12*) also examined individual alkamides and inhibition doses that caused reduction of LPS-mediated NO production in comparison to an LPS control. The ID_{50} for Bauer alkamide 2 was 54 μM, that for Bauer alkamide 8 was 24 μM, that for Bauer alkamide 10 was 40 μM, that for Bauer alkamide 11 was 24 μM, that for Bauer alkamide 13 was 108 μM, that for Chen alkamide 1 was 49 μM, and that for Chen alkamide 2 was 35 μM. The inhibition of NO measured by the ID_{50} corresponds to our PGE_2 data in that our results show significant inhibition of another inflammatory mediator, PGE_2, at 50 μM for all alkamides screened, as well as Bauer alkamide 8 and Chen alkamide 2 significantly inhibiting at 25 μM. Also, in that study (*12*), cytotoxicity sufficient to cause 50% cell death was reported for individual alkamides ranging in concentration from 50 to 217 μM. Only Bauer alkamide 11 showed 50% cell death at 50 μM in the Chen et al. study, which contradicts our results showing Bauer alkamide 11 to produce 94% survival in the Celltiter96 Aqueous One Solution Cell Proliferation Assay at the same concentration. All other alkamides screened by Chen et al. (*12*) were at concentrations higher than those screened by our laboratory for cytotoxicity, perhaps accounting for the cytotoxicity noted in their study.

Studies are providing convincing evidence that alkamides can play important roles in the bioactivity observed in *Echinacea* species, and questions about the bioavailability of this class of constituents are still being answered. Alkamides have been shown to be readily bioavailable through the Caco-2 cell monolayer, more so than other active compounds, such as the caffeic acid derivatives found in *Echinacea* (*31*). Another study had previously supported these data by finding that (2*E*,4*E*,8*Z*,-10*Z*)-*N*-isobutyldodeca-2,4,8,10-tetraenamide could be completely transported from the apical to the basolateral side of the Caco-2 monolayer, with no significant metabolism occurring (*32*). Results from a study investigating the metabolism of natural and synthetic alkylamides from *Echinacea* by using human liver microsomes determined that cytochrome P450 mediated epoxidation, hydroxylation, and dealkylation of alkylamides occurred (*33*). A recent human study analyzed 11 human subjects for bioavailability of an oral administration of a 60% ethanolic extract of *E. angustifolia* root, which was known to contain six identified alkamides, showing that after 30 min one of the six alkamides, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, appeared in plasma samples at

10.88 ng/mL for a 2.5 mL dose (*34*). Another human study analyzed nine volunteers who consumed tablets of 675 mg of *E. purpurea* and 600 mg of *E. angustifolia*, after a high-fat breakfast or after a fast, for alkamide content in their plasma (*35*). Total 2,4-diene alkamides were found in the plasma from the high-fat group with a maximum concentration ranging from 60 to 1137 ng/mL. Although it is becoming increasingly evident that alkamides are bioavailable, more experimentation is warranted to determine whether alkamides can exert anti-inflammatory or other immunomodulatory effects at the low concentration of 10 μM, which was the lowest concentration found to be bioactive in our PGE_2 studies. Without more definitive bioavailablility data, a difficulty arises in making assumptions about alkamides' anti-inflammatory properties based on cell culture data.

In summary, this study indicates that *Echinacea* extracts may be able to modulate inflammation through their inhibitory activity on PGE_2 production and that alkamides are possible key constituents in the observed anti-inflammatory properties, most likely acting additively or synergistically with other constituents. Therefore, because innate concentrations of individual alkamides found in crude extracts do not reach concentrations shown to have significant PGE_2 inhibition capabilities found from tests of pure, synthesized alkamides, it is clear that the presence of individual alkamides alone cannot explain the observed anti-inflammatory activity.

ABBREVIATIONS USED

E., *Echinacea*; PI, Plant Introduction; FNPC, Frontier Natural Products Co-op; NCRPIS, North Central Regional Plant Introduction Station; PGE_2, prostaglandin E_2; LPS, lipopolysaccharide.

SAFETY

Organic solvents, such as hexane and chloroform used for extractions, are toxic chemicals and should be properly handled in a fume hood. LPS compounds are pyrogenic and should not be inhaled or allowed to enter the bloodstream.

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