Echinacea Species and Alkamides Inhibit Prostaglandin E2 Production in RAW264.7 Mouse Macrophage Cells

Carlie A. LaLone  
Iowa State University

Kimberly D.P. Hammer  
Iowa State University

Lankun Wu  
Iowa State University

Jaehood Bae  
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/fshn_ag_pubs

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/fshn_ag_pubs/21. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.
Echinacea Species and Alkamides Inhibit Prostaglandin E2 Production in RAW264.7 Mouse Macrophage Cells

Abstract
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, 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, NCRPIS

Disciplines
Agronomy and Crop Sciences | Cell Biology | Chemistry | Food Science | Genetics | Horticulture | Human and Clinical Nutrition | Pharmacology | Statistics and Probability

Comments

Authors
Carlie A. LaLone, Kimberly D.P. Hammer, Lankun Wu, Jaehood 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

This article is available at Iowa State University Digital Repository: http://lib.dr.iastate.edu/fshn_ag_pubs/21
Echinacea Species and Alkamides Inhibit Prostaglandin E2 Production in RAW264.7 Mouse Macrophage Cells

CARLIE A. LALONE,†,‡,§ KIMBERLY D. P. HAMMER,†,‡,§ LANKUN WU,†,‡ X 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 E2 (PGE2) 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 PGE2 production. In further studies, PGE2 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 PGE2 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 PGE2, 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 abundant 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
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 E2 (PGE2) is a critical inflammatory mediator that is produced through the arachidonic acid cascade. Two cyclooxygenase isozymes, COX-1 and COX-2, catalyze the reaction converting arachidonic acid, released by phospholipase A, to PGE2. LPS is a common endotoxin used to stimulate macrophage cells to produce PGE2, 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 PGE2 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 hydrophilicity 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, shaking with 70% EtOH 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 PGE2 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 PGE2 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 E2.** Echinacea extract and alkamide treatments of RAW264.7 cells and the PGE2 enzyme immunoassay (ELA) used to detect the amount of PGE2 (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 PGE2 production comparing different solvents resulted in significant increases in PGE2 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 Scutel- laria 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 PGE2 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 × 104 cells/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. 

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 media + DMSO control allowing for the combination of treatments above 21 μg/mL. Neither *E. purpurea* accession screened in Figure 1 significantly inhibited PGE2 levels at the 1% concentration (ranging from 240 to 1102 μg/mL) for their ability to reduce PGE2 levels after stimulation with LPS (Figure 1). *E. angustifolia* and *E. pallida* extracts from NCRPIS and Johnny’s Selected Seeds accessions were 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. 

RAW264.7 cells at their highest concentration (ranging in final concentrations from 21 to 53 μg/mL) for their ability to reduce PGE2 levels after stimulation with LPS (Figure 1). *E. angustifolia* and *E. pallida* extracts from NCRPIS and Johnny’s Selected Seeds accessions significantly inhibited PGE2 levels at concentrations above 21 μg/mL. Neither *E. purpurea* accession screened in Figure 1 significantly inhibited PGE2 (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 PGE2 levels in *E. purpurea* and *E. angustifolia* when compared to media + DMSO control. Baicalein and quercetin were included as positive controls for every PGE2 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 extraction 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 PGE2 levels (**, p < 0.05) (Figure 2). Three nonmedicinal species were also screened for anti-inflammatory activity. *E. sanguinea* and *E. simulata* significantly reduced PGE2 production (**, 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 PGE2 production, none was significantly more active than the other when confidence intervals across species were compared.

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 PGE2 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 PGE2 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.
Comparison of extract treatment to control (media + 0.001, for comparison of extract to control (media each), and variability is represented as 95% confidence intervals of the mean. Treatments analyzed without the addition of LPS did not affect PGE2 levels with values for PGE2 as percent of media + DMSO (obtained from NCRPIS with different harvest dates and accession numbers. Each species mean is represented by a bar (two to six replicates without LPS, was excluded from the analysis on the basis of the optical density data point being outside the standard curve.

Table 1. Cytotoxicity Screening of Soxhlet EtOH Extracts from Six Echinacea Species

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

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) 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 PGE2 assay, no significant cytotoxicity remained (p = 0.9968). The screens conducted at 0.1% concentration matched concentrations used in PGE2 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 (≥95 µg/mL of extract), with the exception of one extract showing significant cytotoxicity (p < 0.01) at 39 µg/mL (95% CI 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 PGE2 Production. To further probe the inhibition of PGE2 production in RAW264.7 cells observed with treatments of Echinacea extracts, another PGE2 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 PGE2 (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 PGE2 levels (p < 0.05). Only Bauer alkamide 14 significantly inhibited PGE2 production at 10 µM (p < 0.05). A subsequent PGE2 EIA experiment was conducted to attempt to determine if PGE2 could inhibit at concentrations of <10

Figure 2. Inhibition of PGE2 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 PGE2 levels with values for PGE2 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).

$[^4]$ 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.
Table 2. Inhibition of PGE₂ Production by Alkamides

<table>
<thead>
<tr>
<th>Alkamide Name</th>
<th>Alkamide Structure</th>
<th>50 μM</th>
<th>25 μM</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bauer alkamide 2</td>
<td><img src="image1" alt="Structure" /></td>
<td>55 (30, 71)</td>
<td>32 (-6, 56)</td>
<td>22 (-21, 50)</td>
</tr>
<tr>
<td>Undeca-2Z, 4E-diene-8, 10-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 8</td>
<td><img src="image2" alt="Structure" /></td>
<td>87 (76, 93)</td>
<td>75 (53, 87)</td>
<td>46 (-0.8, 71)</td>
</tr>
<tr>
<td>Dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 10</td>
<td><img src="image3" alt="Structure" /></td>
<td>90 (81, 95)</td>
<td>46 (-2, 71)</td>
<td>42 (-8, 69)</td>
</tr>
<tr>
<td>Dodeca-2E, 4E, 8Z-triеноic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 11</td>
<td><img src="image4" alt="Structure" /></td>
<td>71 (46, 84)</td>
<td>24 (-42, 59)</td>
<td>45 (-3, 71)</td>
</tr>
<tr>
<td>Dodeca-2E, 4E-diеноic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 12</td>
<td><img src="image5" alt="Structure" /></td>
<td>59 (41, 72)</td>
<td>35 (-2, 58)</td>
<td>39 (5, 61)</td>
</tr>
<tr>
<td>Undeca-2E-ene-8, 10-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 12A analogue +</td>
<td><img src="image6" alt="Structure" /></td>
<td>90 (82, 95)</td>
<td>73 (50, 86)</td>
<td>39 (-13, 68)</td>
</tr>
<tr>
<td>Trideca-10, 12-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 12B analogue +</td>
<td><img src="image7" alt="Structure" /></td>
<td>56 (19, 77)</td>
<td>35 (-22, 65)</td>
<td>29 (-32, 62)</td>
</tr>
<tr>
<td>2, 3,4-dihydro Bauer amide 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 13</td>
<td><img src="image8" alt="Structure" /></td>
<td>74 (51, 86)</td>
<td>48 (3, 72)</td>
<td>30 (-32, 62)</td>
</tr>
<tr>
<td>Undeca-2Z-ene-8, 10-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bauer alkamide 14</td>
<td><img src="image9" alt="Structure" /></td>
<td>74 (59, 83)</td>
<td>71 (54, 81)</td>
<td>45 (14, 64)</td>
</tr>
<tr>
<td>Dodeca-2E-ene-8, 10-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chen alkamide 1</strong></td>
<td><img src="image10" alt="Structure" /></td>
<td>74 (51, 86)</td>
<td>32 (-28, 63)</td>
<td>29 (-32, 62)</td>
</tr>
<tr>
<td>Dodeca-2Z, 4E, 10Z-trien-8-yne-10-ynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chen alkamide 1 isomer +</td>
<td><img src="image11" alt="Structure" /></td>
<td>65 (34, 81)</td>
<td>31 (-29, 63)</td>
<td>27 (-37, 61)</td>
</tr>
<tr>
<td>Dodeca-2E, 4E, 10Z-trien-8-yne-10-ynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chen alkamide 2</strong></td>
<td><img src="image12" alt="Structure" /></td>
<td>84 (70, 92)</td>
<td>54 (15, 76)</td>
<td>24 (-42, 59)</td>
</tr>
<tr>
<td>Dodeca-2Z, 4E-diene-8, 10-dynoic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chen alkamide 2 analogue +</td>
<td><img src="image13" alt="Structure" /></td>
<td>89 (80, 94)</td>
<td>78 (59, 88)</td>
<td>40 (-11, 68)</td>
</tr>
<tr>
<td>Dodeca-2Z, 4E-diеноic acid isobutylamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 PGE₂ levels without LPS treatment (data not shown). Bauer alkamides are found in Bauer et al. (14) and **Chen alkamides in Chen et al. (16). 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.
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 PGE2 production were 3.1 and 0.230 μM, and Bauer alkamide 14 was unable to significantly inhibit PGE2 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. 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 PGE2 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. tennesensis contained greater quantities of Bauer alkamides than of ketones or caffeic acid derivatives. Bauer alkamides 3 and 8, which contain similar alkamides, including Bauer alkamide 1 isomer, Chen alkamide 2 analogue, Bauer alkamide 12 at concentrations of >0.1 μM, and Bauer alkamide 13 was present in only certain extracts within the species and are potential among these herbs (Table 3). All Soxhlet EtOH extracts from the six species 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.

DISCUSSION

The results presented in this study demonstrate the inhibition of PGE2 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 PGE2 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 PGE2 in LPS-induced RAW264.7 mouse macrophage cells. E. purpurea extracts (15 μg/mL) did not significantly inhibit PGE2, 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, SW-480 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).

Alkaloids 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). Alkaloids 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). Alkaloids 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 alkaloids are key constituents found in *Echinacea* that possess anti-inflammatory properties. Our experiments indicated that alkaloids are consistent inhibitors of PGE2 production at a concentration of 50 μM, with selected alkaloids having the capability to significantly inhibit PGE2 levels at concentrations of 25 μM or even 10 μM. Although many alkaloids have been identified, it is possible that undescended isomers as well as analogues of many of the known alkaloids 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 PGE2 production by extracts or alkaloids, suggesting that the observed inhibition was a true inhibition of PGE2 and not an artifact due to cell death. The data presented strengthen the research indicating that alkaloids present in *Echinacea* contribute to immunomodulatory properties dealing with regulation of inflammation (27).

Questions as to how alkaloids act together and in concert with other constituents arise from our HPLC analysis. Our data identify alkaloids present in crude *Echinacea* extracts at concentrations below 2.8 μM, which is well under the 10 μM concentration observed for inhibitory activity of PGE2 seen with chemically synthesized Bauer alkamide 14. Regardless, several of the crude extracts of *Echinacea*, containing a variety of alkaloids, ketones, and caffeic acid derivatives at low concentrations, were able to significantly inhibit PGE2 production. For example, *E. sanguinea* extracts at 15 μg/mL, containing Bauer alkaloids 8 and 12–14, at higher concentrations than other constituents present, showed the greatest inhibition of PGE2 among the species we evaluated, with a percent reduction of 59%, whereas none of these alkaloids would be able to inhibit PGE2 production alone at such low concentrations. Therefore, we hypothesize that the noted inhibition of PGE2 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 alkaloids 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 PGE2 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 PGE2 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 PGE2 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 alkaloids 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 alkaloid (a mixture consisting of several alkaloids) ranging from 1.6 to 30 μg/mL. Chen et al. (12) also examined individual alkaloids and inhibition doses that caused reduction of LPS-mediated NO production in comparison to an LPS control. The ID50 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 ID50 corresponds to our PGE2 data in that our results show significant inhibition of another inflammatory mediator, PGE2, at 50 μM for all alkaloids 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 alkaloids 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 alkaloids 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 alkaloids 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. Alkaloids 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 (2E,4E,8Z,10Z)-N-isobutylidodeca-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 alkaloids, showing that after 30 min one of the six alkaloids, dodeca-2E,4E,8Z,10E/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 (36), total 2,4-diene alkamides were found in the plasma from tests of pure, synthesized alkamides, it is clear that the presence of individual alkamides alone cannot explain the observed anti-inflammatory activity.

In summary, this study indicates that *Echinacea* extracts may be able to modulate inflammation through their inhibitory activity on PGE2 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 PGE2 inhibition capabilities found from tests of pure, synthesized alkamides, it is clear that the observed anti-inflammatory activity based on cell culture data.

**ABBREVIATIONS USED**

*E., Echinacea; PI, Plant Introduction; FNPC, Frontier Natural Products Co-op; NCRPIS, North Central Regional Plant Introduction Station; PGE2, prostaglandin E2; 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.

**ACKNOWLEDGMENT**

We thank all members of the Center for Research on Botanical Dietary Supplements at Iowa State University and the University of Iowa for their cooperation and ongoing advice in directing the progress of this research. We give special thanks for the gift of *Echinacea* plant material from Frontier Natural Products Co-op (Norway, IA) as well as to Fredy Romero and members of the Organic Agriculture Program and the NCRPIS at Iowa State University (Ames, IA). We acknowledge Philip Dixon and Man-Yu Yum for their statistical guidance as well as Zili Zhai and Joan Cunnick for endotoxin analysis of the NCRPIS extracts.

**LITERATURE CITED**


