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Endogenous levels of *Echinacea* alkylamides and ketones are important contributors to the inhibition of prostaglandin E2 and nitric oxide production in cultured macrophages

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Abstract

Due to the popularity of *Echinacea* as a dietary supplement, researchers have been actively investigating which *Echinacea* constituent or groups of constituents are necessary for immune modulating bioactivities. Our prior studies indicate that alkylamides may play an important role in the inhibition of prostaglandin E2 (PGE₂) production. HPLC fractionation, employed to elucidate interacting anti-inflammatory constituents from ethanol extracts of *E. purpurea*, *E. angustifolia*, *E. pallida*, and *E. tennesseensis* identified fractions containing alkylamides and ketones as key anti-inflammatory contributors using lipopolysaccharide induced PGE₂ production in RAW264.7 mouse macrophage cells. Nitric oxide (NO) production and parallel cytotoxicity screens were also employed to substantiate an anti-inflammatory response. *Echinacea pallida* showed significant inhibition of PGE₂ with a first round fraction, containing GC-MS peaks for Bauer Ketones 20, 21, 22, 23, and 24, with 23 and 24 identified as significant contributors to this PGE₂ inhibition. Chemically synthesized Bauer Ketones 21 and 23 at 1 μM each significantly inhibited both PGE₂ and NO production. Three rounds of fractionation were produced from an *E. angustifolia* extract. GC-MS analysis identified the presence of Bauer Ketone 23 in third round Fraction 3D32 and Bauer Alkylamide 11 making up 96% of third round Fraction 3E40. Synthetic Bauer Ketone 23 inhibited PGE₂ production to 83 % of control and synthetic Bauer Alkylamide 11 significantly inhibited PGE₂ and NO production at the endogenous concentrations determined to be present in their respective fraction, thus each constituent partially explained the *in vitro* anti-inflammatory activity of their respective fraction. From this study two key contributors to the anti-inflammatory properties of *E. angustifolia* were identified as Bauer Alkylamide 11 and Bauer Ketone 23.

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Safety

LPS compounds are pyrogenic and should not be inhaled or allowed to enter the bloodstream.

Keywords

Echinacea purpurea; *Echinacea angustifolia*; *Echinacea pallida*; *Echinacea angustifolia*; Prostaglandin E2; Nitric Oxide; Bauer Alkylamides; Bauer Ketones; Anti-inflammatory; Fractionation

Introduction

Sales of *Echinacea* as a botanical supplement have remained high over recent years in the United States reaching approximately twenty-one million dollars in 2005 (1). The efficacy and health benefits of taking *Echinacea* as a supplement have yet to be verified scientifically and researchers are still unclear as to how the constituents of *Echinacea* act individually or in concert to elicit the bioactive properties that have been observed in numerous studies, both *in vitro* and *in vivo* (2,3). Although *Echinacea* extracts are complex mixtures consisting of several constituents, alkylamides and caffeic acid derivatives have received considerable attention recently for their abilities to modulate the immune system. Recent studies have shown that alkylamides of *Echinacea* are partially responsible for anti-inflammatory responses such as inhibition of PGE₂, TNF- α , and NO production in RAW264.7 mouse macrophage cells (2–5), as well as inhibition of cyclooxygenase activity in neuroglioma cells and other *in vitro* model systems (4,6). Studies have further validated that alkylamides are capable of binding to and activating the cannabinoid receptor type-2 providing insight into the mechanism by which these constituents may modulate immune function (7,8). Caffeic acid derivatives have been associated with anti-viral and anti-oxidant properties (9–11). It has been hypothesized that alkylamides and caffeic acid derivatives interact, perhaps synergistically, with each other or other compounds to elicit immunomodulatory effects (9).

Prostaglandin E2 is a major lipid mediator of inflammation that is produced through the activation of the arachidonic acid cascade, via the enzymatic activity of the cyclooxygenase isoforms. The inducible nature of PGE₂ production when macrophage cells are stimulated by lipopolysaccharide (LPS) makes this eicosinoid an ideal target for measuring an inflammatory response *in vitro*.

Our studies were conducted to identify *Echinacea* constituents that are responsible for the previously described PGE₂ inhibition (2). Methods have been developed to quantitatively determine the amount of alkylamides and caffeic acid derivatives present in different parts of the *Echinacea* plant using reverse phased HPLC and GC-MS analysis (12,13). Semi-preparative reverse phased HPLC was utilized to fractionate *Echinacea* extracts into fractions and sub-fractions that separate phytochemicals according to their hydrophobic properties, concentrating a reduced number of constituents to analyze for anti-inflammatory potential. Eluents from HPLC fractionations were monitored for absorbance at wavelengths of 260 nm and 330 nm in order to identify lipophilic alkylamides and phenolic compounds, such as caffeic acid derivatives. Further fractionation was guided by identifying fractions capable of inhibiting PGE₂ production, allowing for a thorough investigation into the hypothesized synergistic or additive interactions that are thought to occur among the constituents of *Echinacea* extracts and allow for the identification of key anti-inflammatory constituents through the use of GC-MS analysis. In order to have a more complete view of how interacting constituents found in *Echinacea* inhibit inflammatory mediators, NO production was also assessed in RAW264.7 macrophage cells treated with chemically synthesized phytochemicals, which were identified to be important in the PGE₂ assay with *E. angustifolia*.

Materials and Methods

Plant Material and Extraction

Plant materials were provided by the USDA North Central Regional Plant Introduction Station (NCRPIS, Ames, IA). *E. angustifolia* (PI631285), *E. purpurea* (PI631307), *E. pallida* (PI631293), *E. tennesseensis* (PI631250) were used for the semi-preparative HPLC fractionation. Root material from each species was collected as previously described (2) from a 2006 harvest. Further information about the accessions can be found on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html provided by NCRPIS. Plant materials were stored at -20°C under nitrogen in zip-lock bags prior to use. The *Echinacea* plant materials were all dried root powders. They were previously washed then completely dried at 40°C forced air conditions, followed by grinding through a 40-mesh screen Wiley grinder (14).

For each accession, 6 grams of *Echinacea* root material was extracted with 95 % ethanol and 5 % endotoxin-free water using Soxhlet apparatus for 6 hours for exhaustive extraction, following the protocol created by Liu (15). To avoid endotoxin contamination the glassware was heated to 180°C for at least 2 hours prior to use. After the extraction, the 95 % aqueous ethanol solvent was evaporated using a Rotavap (Buchi rotavaps model R-144, R-110, R-111, and R-200, Switzerland) to obtain the dried extract, which was weighed. The dried extracts were redissolved in endotoxin-free water, and ethanol was added at a ratio of 1:3 (water: ethanol) to obtain a concentration of no more than 0.6 g of extracted material/ml for a 5 ml injection through the semi-preparative HPLC fractionation. The extracts were stored at -20°C overnight prior to fractionation.

Semi-Preparative HPLC Fractionation

The 95 % ethanol extracts of *Echinacea* were filtered through a $0.45\ \mu\text{m}$ filter prior to injection into the semi-preparative HPLC system consisting of two Beckman Model 110B pumps controlled by a Module406 Beckman System Gold Analogue Interface (Beckman Coulter, Fullerton, CA) using a YMC-PACK ODS-AM 250*10 mm I.D. s- $5\ \mu\text{m}$, 12 nm reversed phase C18 columns (Waters Corp.) and a 5 ml loop on a Rheodyne model 7010 injection valve.

Fractionation was conducted with a solvent gradient designed with acetonitrile as solvent B and 0.1 % HPLC grade glacial acetic acid in endotoxin-free Milli-Q water as solvent A. The gradient started at 10 % B, with a flow rate of 3 ml/min, and increased to 30 % B in 30 min. At 30 min, the gradient increased to 90 % B over 50 min. At 80 min, the gradient increased to 100 % B in 10 min. The gradient was held at 100 % B for 20 min. Fraction 1 was collected in the first 30 min of the gradient. Fraction 2 was collected between 30 and 40 min; fraction 3 was collected between 40 and 80 min; fraction 4 was collected between 80 and 90 min; and fraction 5 was collected between 90 and 110 min. Second round fractions were generated by collecting at 1 min intervals across the same gradient profile within bioactive fractions. For bioassay purposes, subfractions of *E. angustifolia* Fraction 3 (3A, 3B, 3C, 3D, and 3E) were created by pooling 8 min intervals across the 40 min of Fraction 3. For example, 3B represents eluent collected between min 9 and min 16 within Fraction 3 (or at min 49 to min 56 of gradient time). The third round fractions were then collected at 1 min intervals. For example, Fraction 3D28 was the 3 ml fraction collected at 28 minutes into Fraction 3 (or at min 68 of gradient time). The fractions and subfractions were dried, first by removal of the organic phase by rotary evaporation, and second, by the amount of water by freeze-drying. Compounds in first round fractions of all *Echinacea* species and second round fractions produced from *E. angustifolia* were identified using HPLC compared to synthetic standards. The literature has shown that alkylamides dissolved in liquid form and stored at -20°C are stable over extended periods of

time (15–17). Therefore, all fractions were diluted in dimethyl sulfoxide (DMSO, Sigma; St. Louis, MO) and stored at -20°C to maintain the stability of alkylamides and thawed at room temperature in preparation for analysis.

High Performance Liquid Chromatography Analysis of *E. angustifolia* Second Round Fractions

HPLC analysis and quantification of constituents found in second round fractions of *E. angustifolia* was performed as previously described (2).

GC-MS Analysis

GC-MS analyses were performed using an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph (6890 series), equipped with a model 5973 mass detector operating in the electron impact ionization mode (70 eV). The analyses were carried out by injecting in splitless mode. Analytes were separated using an Agilent Technologies capillary column (HP-5MS fused silica column coated with 5% diphenyl 95% dimethyl polysiloxane, with the dimensions 30 m length \times 250 μm bore, 0.25 μm film thickness). Helium was used as the carrier gas at the flow rate of 1.2 ml/min. Identification of compounds was facilitated by using the Agilent Technologies enhanced ChemStation Software, version D.02.00.275.

Alkylamide and Ketone Synthesis

Alkylamide synthesis was conducted as described previously (2). Ketones were chemically synthesized according to the procedures outlined by Kraus et al. (18) and in the thesis of Jaehoon Bae (19). Alkylamide and ketone concentrations were calculated after correcting for percent purity, yielding concentrations equivalent to 100 % pure synthetic constituent. Calculated percent purity before correction for Bauer Alkylamide 10 was 82 %, Bauer Alkylamide 11 was 92 %, Bauer Ketone 20 was 82 %, Bauer Ketone 21 was 76 %, Bauer Ketone 23 was 90 %, and Bauer Ketone24 was 99%. All synthetic alkylamides and ketones were stored at -80°C under argon gas.

Cell Culture

The cell culture model used for these studies was RAW264.7 mouse monocyte/macrophage cells that were obtained from American Type Culture Collection (cat: TIB-71, Manassas, VA). All culturing conditions and procedures were previously described by Hammer et al. (20) with the exception of maintaining optimal growth conditions at 95% humidity.

Measurement of Prostaglandin E₂

Prostaglandin E₂ production was analyzed using PGE₂ enzyme immunoassay (GE Biosciences; Piscataway, NJ) after treating RAW264.7 mouse macrophage cells for eight hours with fractions from *E. angustifolia*, *E. purpurea*, *E. pallida*, and *E. tenesseensis* and with or without lipopolysaccharide (*E. coli* O26:B6, Sigma; St. Louis, MO) as previously described (2). Quercetin (3,5,7,3'4'-pentahydroxy flavon), a common flavanoid found in many plant species including *Echinacea*, was used as the positive control at a concentration of 10 μM (Sigma; St. Louis, MO). Also, baicalein (5,6,7-trihydroxyflavone), a flavanoid found in the medicinal herb *Scutellaria baicalensis* was used as a positive control at a concentration of 6 μM (Synthesized by G. A. Kraus's laboratory at Iowa State University).

Measurement of Nitric Oxide Production

Nitric oxide production was analyzed using Griess Reagent System (Promega; Madison, WI) following the manufacturers protocol. RAW264.7 cells were plated at a density of 1.57×10^5 cells/well in a 24 well cell culture plate and incubated overnight. Chemically synthesized

ketones of *Echinacea* and combinations of alkylamides and/or ketones were then added to the cells either with or without LPS for a 24 hour time period. Each treatment contained four controls which were media, media + DMSO (vehicle control), media + LPS, and media + DMSO + LPS. Quercetin was used as the positive control at a concentration of 10 μM (Sigma; St. Louis, MO). After the 24 hour treatment period, the cell supernatants were collected, stored at 4°C until used in the NO assay.

Cytotoxicity of *Echinacea* Fractions

The method used to detect cytotoxicity was previously described by LaLone et al. (2) using the Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Cytotoxicity analysis was carried out for all fractions from all *Echinacea* species analyzed. Each fraction was screened for cytotoxicity at concentrations comparable to those used in the PGE₂ assay and incubated for 24 hours. Ursolic acid (Fisher Scientific; Hanover Park, IL) was used as the positive control at three concentrations, 10 μM , 30 μM , and 50 μM , with significant cytotoxicity identified at the two highest concentrations. Synthetic Bauer Alkylamide 11 and Bauer Ketone 23 were also screened for cytotoxicity after incubation with macrophages for 72 hours.

Statistical Analysis

Both log transformed PGE₂ data and NO data were analyzed using randomized complete block design with variable levels of treatment, followed by a t-test based on pooled error variance to determine statistical significance compared to the (Media + DMSO + LPS) control. In all figures, the data are represented as % of control \pm standard error, normalizing the (Media + DMSO + LPS) control to 100% PGE₂ or NO production within each block and summarizing across all blocks to obtain the mean and standard error. The three subsamples of cytotoxicity values in each block were averaged before analysis as a randomized complete block design as above. The cytotoxicity data are also presented as % of control \pm standard error, normalizing the (Media + DMSO) control to 100% cell survival. All statistical analysis was conducted using the GLM procedures in SAS (version 9.1, SAS Institute Inc.; Cary, NC).

Results

Fractions from *E. pallida*, *E. purpurea*, and *E. tennesseensis* Inhibit PGE₂ Production

In order to elucidate key constituents capable of inhibiting select inflammatory mediators, a Soxhlet ethanol extract of *E. pallida* was fractionated into five fractions and each was assessed for its effect on PGE₂ production in RAW264.7 mouse macrophage cells. The analysis yielded one fraction, Fraction 3, capable of significantly inhibiting PGE₂ production (36.8 % of control) at a concentration of 5 $\mu\text{g}/\text{ml}$ (Figure 1). Another interesting observation was that Fraction 3, at a concentration of 5 $\mu\text{g}/\text{ml}$, inhibited PGE₂ production to a greater extent than the initial ethanol extract of *E. pallida* at a concentration of 25 $\mu\text{g}/\text{ml}$, indicating the importance of enriching selected constituents and separating them from others using fractionation. When Fraction 3 from *E. pallida* was diluted from 5 $\mu\text{g}/\text{ml}$ to 1 $\mu\text{g}/\text{ml}$, inhibition of PGE₂ production was still observable (71.2 % of control, $p = 0.07$). In a parallel cytotoxicity study it was determined that the fractions from *E. pallida* were not cytotoxic to the RAW264.7 mouse macrophage cells, indicating that cell death could not account for the observed inhibition of PGE₂ production (Figure 1) GC-MS analysis indicated that Fraction 3 contains numerous constituents, including Bauer Ketones 20, 21, 22, 23, and 24 (Figure 2A). Quantification of Bauer Ketones, 20, 21, 23, and 24 present in Fraction 3 yielded concentrations of 0.7 μM , 0.6 μM , 2.6 μM , and 5 μM , respectively. Combinations of quantified Bauer Ketones were analyzed for their ability to inhibit PGE₂ production (Figure 2B). This analysis indicated that Bauer Ketones 23 and 24 were the most important of the identified ketones in partially explaining the PGE₂ inhibitory capabilities of Fraction 3 from *E. pallida*. Chemically synthesized Bauer

Ketones 20, 21, and 23, which are constituents of *E. pallida* ethanol extracts and HPLC generated fractions (Figure 3A), were analyzed for their ability to inhibit PGE₂ production and NO production at three concentrations (5 μM, 2.5 μM, and 1 μM) to determine if they played a key role in the significant inhibition of PGE₂ identified with Fraction 3. It was determined that Bauer Ketones 21 and 23 were capable of significant inhibition of PGE₂ (p<0.05) and NO (p<0.0001) production at a concentration as low as 1 μM (Figure 3B).

Fractionation of a Soxhlet ethanol extract of *E. purpurea* yielded seven fractions, none of which were able to significantly inhibit PGE₂ production and none were found to be cytotoxic (Table 1).

A Soxhlet ethanol extract of *E. tennesseensis*, which is a less studied species, was fractionated into five fractions by reverse phase HPLC in order to identify PGE₂ inhibitory capabilities. Fraction 3, containing Bauer Alkylamides 12, 13, 14, 16, and 17, was able to significantly inhibit PGE₂ at a concentration of 41 μg/ml or 20 μg/ml, but when the fraction was diluted to 5 μg/ml its inhibitory ability was lost (Table 1). The initial ethanol extract (59 μg/ml) and HPLC Fraction 5 (23 μg/ml) of *E. tennesseensis* were significantly cytotoxic to the RAW264.7 cells, but due to their inability to significantly inhibit PGE₂ production further dilutions were not carried out.

Three Rounds of Bioactivity Guided Fractionation of an *E. angustifolia* Ethanol Extract

Inhibition of PGE₂ production was used to guide three rounds of HPLC fractionation initiated with a Soxhlet ethanol extract of *E. angustifolia*. Figure 4 outlines the fractionation scheme. The first round of fractionation produced 5 fractions. The alkylamide rich Fraction 3, from *E. angustifolia*, was shown to significantly inhibit PGE₂ production (51.1 % of control) at a concentration as low as 1 μg/ml (Figure 5). Although fraction 4 had the ability to significantly inhibit PGE₂ production at a concentration of 16 μg/ml, it proved to be significantly cytotoxic for the RAW264.7 cells. Therefore, Fraction 3 was identified for further rounds of fractionation.

The second round of fractionation, initiated with Fraction 3 from the previous round, yielded five second-round fractions labeled 3A through 3E. Of these, Fractions 3D and 3E, at a concentration of 5 μg/ml, significantly inhibit PGE₂ production at 47.1 % and 38.6 % of control, respectively, without any obvious cytotoxicity (Figure 6A). HPLC analysis of solids from 0.15 mg second round fractions 3D and 3E led to the detection of several alkylamides (as detected by absorbance at 260 nm) in Fraction 3D (Figure 6B), and identification of Bauer Alkylamides 10 and 11 in Fraction 3E (Figure 6C). Concentrations of Bauer Alkylamides 10 and 11 were estimated in Fraction 3E from the HPLC analysis at 5.62 and 9.48 μM, respectively (Table 2). PGE₂ production was analyzed to determine whether these chemically synthesized Bauer Alkylamides, individually or in combination at the concentrations detected in Fraction 3E were able to explain the inhibition of PGE₂ production observed with Fraction 3E. The combination of Bauer Alkylamides 10 and 11 at the concentrations detected by the HPLC analysis of Fraction 3E, inhibited PGE₂ production 82.6% of control, which indicates therefore that the two alkylamides could not be the sole components responsible for the observed bioactivity of fraction 3E (Figure 7). In these experiments, cell viability was not significantly different than the media + DMSO control. It was interesting to note that synthetic Bauer Alkylamides 10 and 11 when screened alone in the PGE₂ production assay, were significantly inhibitory at the concentrations found in Fraction 3E, but additive effects were not detected in the PGE₂ production screen. The combination of alkylamides found in Fraction 3D was not carried out as Fraction 3E was more effective at inhibiting PGE₂ production and there were several other alkylamides identified in Fraction 3D, many of which are not synthetically available.

A third round of HPLC fractionation was carried out starting with the material from second round fractions 3D and 3E to further elucidate key constituents found in *E. angustifolia* that contribute to the inhibition of PGE₂ production. Fractionation of Fractions 3D and 3E each produced eight fractions labeled Fractions 3D25 through 3D32 and Fractions 3E33 through 3E40, respectively. One of these, Fraction 3D32 was significantly inhibitory of PGE₂ production (44.9 % of control) at a concentration of 1 µg/ml (Figure 8A). The fractionation of Fraction 3E produced two fractions that significantly inhibited PGE₂ production at a concentration of 1 µg/ml, Fraction 3E33 that inhibited PGE₂ production at 14.1 % of control, and Fraction 3E40 that inhibited PGE₂ production at 18.4 % of control (Figure 8B). These third round fractions were not significantly cytotoxic to the RAW264.7 mouse macrophage cells.

GC-MS Analysis of Selected Third Round *E. angustifolia* Fractions

Selected fractions from the third round fractionation of *E. angustifolia* were analyzed by GC-MS based on their activity in the PGE₂ production assay (Table 3). Fractions 3D32, 3E33 and 3E40 were selected for GC-MS analysis due to their ability to significantly inhibit PGE₂ production at a concentration of 1 µg/ml. Two fractions, Fraction 3D28 and 3E38, were also selected for further analysis because they were fractions that did not significantly inhibit PGE₂ production. These analyses, determined that Bauer Ketone 23 is a major constituent of Fraction 3D32, occurring at a concentration of 0.83 µM. Bauer Ketone 23 and Bauer Alkylamide 10 were detected in 1 µg/ml of Fraction 3E33 at concentrations of 0.15 µM and 0.25 µM, respectively and Bauer Alkylamide 11 was quantified to be present at a concentration of 3.55 µM in Fraction 3E40.

Combinations of synthetic constituents and individual synthetic constituents, identified to be present through GC-MS analysis of these third round HPLC fractions were screened for their ability to modulate PGE₂ and NO production at the concentrations present in their respective third round fractions. Synthetic Bauer Ketone 23 was shown to significantly inhibit PGE₂ and NO production previously at a concentration of 1 µM (Figure 3B), and after further dilution of Bauer Ketone 23 to the concentration of 0.83 µM inhibition of PGE₂ production remained significant whereas NO production was not significantly inhibited (55.2 % of control and 68.2% of control, respectively) (Figure 9 for PGE₂ data). Bauer Ketone demonstrated no cytotoxicity after incubation for 72 hours (109.1% of control). Synthetic Bauer Alkylamide 11, at a concentration of 3.55 µM significantly inhibited PGE₂ production (70.6 % of control) and NO production (75.1 % of control) (Figure 10), without any cytotoxicity after a 72 hour incubation (94.1% of control). The combination of synthetic Bauer Alkylamide 10 and Bauer Ketone 23 did not inhibit PGE₂ production at the concentrations present in Fraction 3E33 (121.0 % of control). Neither synthetic Bauer Ketone 23 alone nor the combination of Bauer Alkylamide 10 and Bauer Ketone 23 at the concentrations present in the respective bioactive fractions significantly modulated NO production or cell viability ($p \geq 0.09$).

Although identified alkylamides and ketones found in these bioactive fractions were stable under our storage and experimental conditions (analyzed by GC-MS), instability of bioactivity was identified with these third round bioactive fractions of *E. angustifolia*. Thus, although these fractions were significantly bioactive when initially assayed soon after fractionation, in February 2007, when these fractions were re-assayed for bioactivity when the synthesis of Bauer Alkylamides and Ketones became available in 2008, these fractions did not retain the same level of inhibitory activity in the PGE₂ production assay at the concentrations previously analyzed (Table 4).

Discussion

The study reported here illustrates the important role *Echinacea* alkylamides and ketones play in the inhibition of the production of inflammatory mediators and the complexities associated

with the examination of these interacting constituents. The significant discoveries from the present study are the identification of Bauer Ketones 21 and 23 as potential anti-inflammatory agents capable of significant inhibition of PGE₂ and NO production at 1 μM concentrations and the finding that chemically synthesized Bauer Ketone 23 and Bauer Alkylamide 11 when screened for inhibition of PGE₂ production at concentrations present in their respective *E. angustifolia* fractions, are capable of inhibiting and partially explaining the significant PGE₂ suppression identified with their respective plant derived fractions. Also, Bauer Ketones 23 and 24 were identified as key components responsible for the inhibition of PGE₂ identified with *E. pallida* Fraction 3. Another key observation is that caffeic acid derivatives found in *Echinacea* are not likely to be key contributors to the inhibition of certain inflammatory mediators due to the fact that these constituents were concentrated in Fraction 1 of all of the species fractionated, and none of these fractions demonstrated the ability to significantly inhibit PGE₂ production at concentrations ranging from 26 μg/ml to 329 μg/ml.

Ethanol extracts from *E. tennesseensis* and *E. purpurea* were unable to produce fractions capable of inhibiting PGE₂ production to a significant degree at concentrations below 5 μg/ml. Therefore, studies on these two species ceased after the first round of fractionation.

Of the four species screened *E. pallida* and *E. angustifolia* were expected to produce the most active fractions because of previous results (2) indicating that extracts from these plants were inhibitory in the PGE₂ assay. The fractionation conducted with a ketone rich accession of *E. pallida* provided insight into the anti-inflammatory potential of ketones found in this species. Compared to our previous studies that examined chemically synthesized alkylamides and their ability to inhibit PGE₂ production (2), it appears that ketones are able to significantly inhibit this inflammatory endpoint at a much lower concentration. For example, the most potent alkylamide (Bauer Alkylamide 14) was reported to significantly inhibit PGE₂ production, at a concentration of 10 μM (2), whereas Bauer Ketone 23 could do so at a concentration of 1 μM. Significant cytotoxicity was not identified for any of the Bauer Ketones screened at concentrations below 20 μM, coinciding with a recent cell viability study reporting that Bauer Ketone 21 had IC₅₀ values of >100 μM and 80.13 μM in human pancreatic and colorectal adenocarcinoma cells, respectively (10). By examining the ability of selected polyacetylenes isolated from n-hexane extracts of *E. pallida* to cross the Caco-2 monolayer, this study also provided evidence that Bauer Ketones 22 and 24 are likely to be bioavailable, with apparent permeabilities of $32 \times 10^{-6} \text{ cms}^{-1}$ and $10 \times 10^{-6} \text{ cms}^{-1}$, respectively (10). Therefore, these results warrant further elucidation with ketones found in other species of *Echinacea* to identify their full anti-inflammatory potential.

Previous studies have shown that *Echinacea* polyacetylenes, generically called ketones, were able to modulate the multidrug transporter P-glycoprotein (Pgp), which bestows resistance to anticancer chemotherapy when highly expressed in cancer cells. In a human kidney cell line (HK-2), made to constitutively over express high and constant levels of Pgp, it was determined through a bioassay-guided fractionation of an *E. pallida* extract that Bauer Ketone 24 decreased the efflux of the Pgp probe calcein-AM by three-fold compared to the control at a concentration of 30 μM (21). To our knowledge there are no reports regarding the anti-inflammatory potential of the Bauer Ketones, yet there are studies describing anti-inflammatory effects of polyacetylenes in other species. For example, polyacetylenes of *Daucus carota L.* have been shown to inhibit LPS induced nitric oxide production in the RAW264.7 macrophage cell line (22) and polyacetylene spiroketals from *Plagius flosculosus* have been identified to inhibit LPS induced IL-1, IL-6, TNF-α, and PGE₂ production, as well as inhibit the degradation of IκB and further DNA binding of NF-κB (23).

Echinacea angustifolia, classified as one of the three *Echinacea* medicinal species, has been featured in other studies due to its alkylamide rich composition (2,24,25). The first round of

HPLC fractionation of the ethanol extract of *E. angustifolia* yielded one fraction, Fraction 3, which inhibited PGE₂ production by 51.1% of control at a concentration of 1 µg/ml, therefore establishing a rationale for additional fractionation. Results from the second round of HPLC fractionation yielded two fractions, Fraction 3D and 3E, which significantly inhibited PGE₂ production. Multiple alkylamides were identified in Fraction 3D, which made this fraction an excellent candidate for further fractionation.

HPLC analysis of Fraction 3E indicated the occurrence of two highly abundant alkylamides, Bauer Alkylamides 10 and 11, which had previously been chemically synthesized for our studies (2). We hypothesized that Bauer Alkylamides 10 and 11, when combined at concentrations similar to those found in Fraction 3E, could explain that fractions PGE₂ inhibitory capabilities. Synthetic Bauer Alkylamide 10 and 11 individually were capable of significant inhibition of PGE₂ production at concentrations of 5.6 µM and 9.5 µM, respectively. However, the combination of synthesized Bauer Alkylamides 10 and 11 at concentrations present in Fraction 3E could not explain Fraction 3E's PGE₂ inhibitory capabilities. Previous studies in our lab found that individually, chemically synthesized Bauer Alkylamide 10 and 11 were not capable of significant PGE₂ inhibition at concentrations lower than 50 µM. We attribute this significant change in activity to a couple of modifications in our screening protocol. The first major change was using optimal growth conditions for the RAW264.7 cells by setting the incubation humidity to 95% as opposed to our previous studies that used 70% humidity. Second, in the current study we analyze each chemically synthesized preparation prior to its use as an inhibitor of PGE₂ production, allowing for impurities in the alkylamide preparations, and normalizing to a concentration at 100% purity; this normalization was not conducted in our previous studies of the inhibition of PGE₂ production. These changes have therefore allowed for greater sensitivity in our screening of inflammatory mediators using the RAW264.7 mouse macrophage cell line.

GC-MS analysis of the third round of HPLC fractions of *E. angustifolia* identified Bauer Ketone 23 to be present in two of these fractions; Fraction 3D32 contained Bauer Ketone 23 at a concentration of 0.83 µM, and several other unidentifiable peaks. To our knowledge this is the first study to identify the presence of Bauer Ketone 23 in *E. angustifolia*. Bauer Ketone 23 partially explained the inhibition of PGE₂ production observed with Fraction 3D32 (44.9 % of control) and can therefore be identified as a key constituent contributing to the immune modulating properties of *E. angustifolia* and perhaps other species. Chemically synthesized Bauer Alkylamide 11 showed significant inhibition of PGE₂ and NO production at the concentration that this alkylamide occurs in Fraction 3E40. Although this constituent appeared to account for approximately 96% of the mass that occurred in this fraction, it only partially explained the PGE₂ inhibition observed with its respective fraction. Through these studies, Bauer Alkylamide 11 of *E. angustifolia* was identified as another key contributor to the suppression of PGE₂ and NO. This alkylamide was shown to have bioactivities at concentrations relevant to those found in the plant extracts and contributed to anti-inflammatory properties throughout an inflammatory response measured via PGE₂ production at eight hours and nitric oxide production at 24 hours after induction with LPS to induce the mouse macrophage cells. Chen et al. previously identified Bauer Alkylamide 11 as an inhibitor of NO production in the RAW264.7 cells with an ID₅₀ of 23.9 µM (3) and our studies add to this by determining that inhibition of NO production can be accomplished at alkylamide concentrations available in *E. angustifolia*.

Two main observations from these studies have led us to the hypothesis that other unidentified constituents found in *Echinacea* are critical components to the anti-inflammatory potential of this botanical. First we have demonstrated that individually Bauer Alkylamides and Ketones can significantly inhibit PGE₂ and NO production, but only partially explain the activity found in the fractions from the species. Also, when known constituents were combined at

concentrations relevant to extracts and partially purified fractions there was no evidence of an additive or synergistic effect on inhibition of PGE₂ or NO production. Secondly, although the concentrations of Bauer Alkylamide 10 and 11, and Bauer Ketone 23 did not change over storage time throughout our studies, the most convincing argument for this hypothesis developed through the instability we observed throughout our studies with the bioactivity of *E. angustifolia* third round fractions. Significant inhibition of PGE₂ production was lost after storing selected third round fractions at -20°C for approximately one year, without any significant decrease in the concentrations of Bauer Alkylamides and Ketones. These observations provide evidence to the hypothesis that other unstable constituents contribute to the identified inhibition of PGE₂ production

In summary, this research confirmed our previous studies that *E. angustifolia* and *E. pallida* are important species of *Echinacea* for discovering the anti-inflammatory properties of this botanical genus, and further allowed for the identification of constituents that are key contributors to those properties. From the fractionations of *E. pallida* and *E. angustifolia* extracts two major compounds, Bauer Alkylamide 11 and Bauer Ketone 23, were identified to play a key role in the inhibition of PGE₂ by RAW264.7 mouse macrophage cell model. These constituents were also identified as inhibitors of NO production, indicating that they are important mediators for an extended period throughout the inflammatory response. The analysis of Bauer Ketone 23 at the concentration present in Fraction 3D32, and of Bauer Alkylamide 11 at the concentration present in Fraction 3E40, indicates that more studies should be directed toward the identification and synthesis of known and unknown compounds and the roles they may play, either individually or in concert with known constituents, to modulate mediators of the inflammatory response.

The results obtained from this study may pave the way for the production of *Echinacea* species that are better suited for anti-inflammatory medicinal purposes. These could be produced through the fabrication of genetically modified plants, the detection of other plant organs and tissues that are rich in specific constituents, or the identification of optimal growth conditions for the enhanced availability of constituents such as Bauer Ketones 21, 23, and 24 and Bauer Alkylamides 10 and 11.

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Abbreviations Used

E	<i>Echinacea</i>
PI	Plant Introduction
DMSO	Dimethyl Sulfoxide
PGE₂	Prostaglandin E2

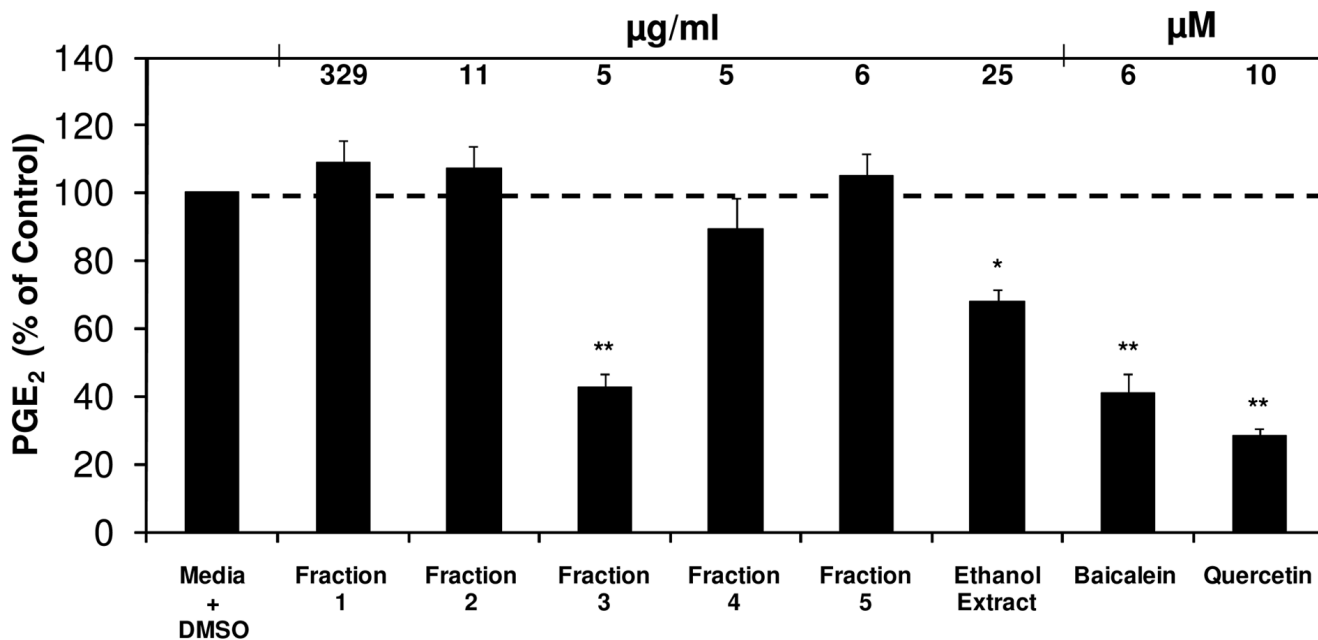
NO	Nitric Oxide
LPS	Lipopolysaccharide
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
ID₅₀	Inhibition Dose producing 50 % inhibition

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A

**Figure 1.**

(A) Fraction 3 from a 2005 extract of *Echinacea pallida* (PI631293) significantly inhibited PGE₂ production in RAW264.7 cells. The black bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction or ethanol extract (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % (2.0 ng/ml). Treatments were also performed without LPS induction showing no significant reduction in PGE₂ production (p ≥ 0.21). The treatments without LPS were compared to the media + DMSO control set at 100 % (0.2 ng/ml). Media alone did not inhibit PGE₂ production (104 % of control). Baicalein and quercetin were used as positive controls and showed significant inhibition of PGE₂ production (p < 0.001). Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the *E. pallida* fractions (data not shown). * and ** are representative of p < 0.05 and p < 0.001. Each bar represents % of control ± standard error.

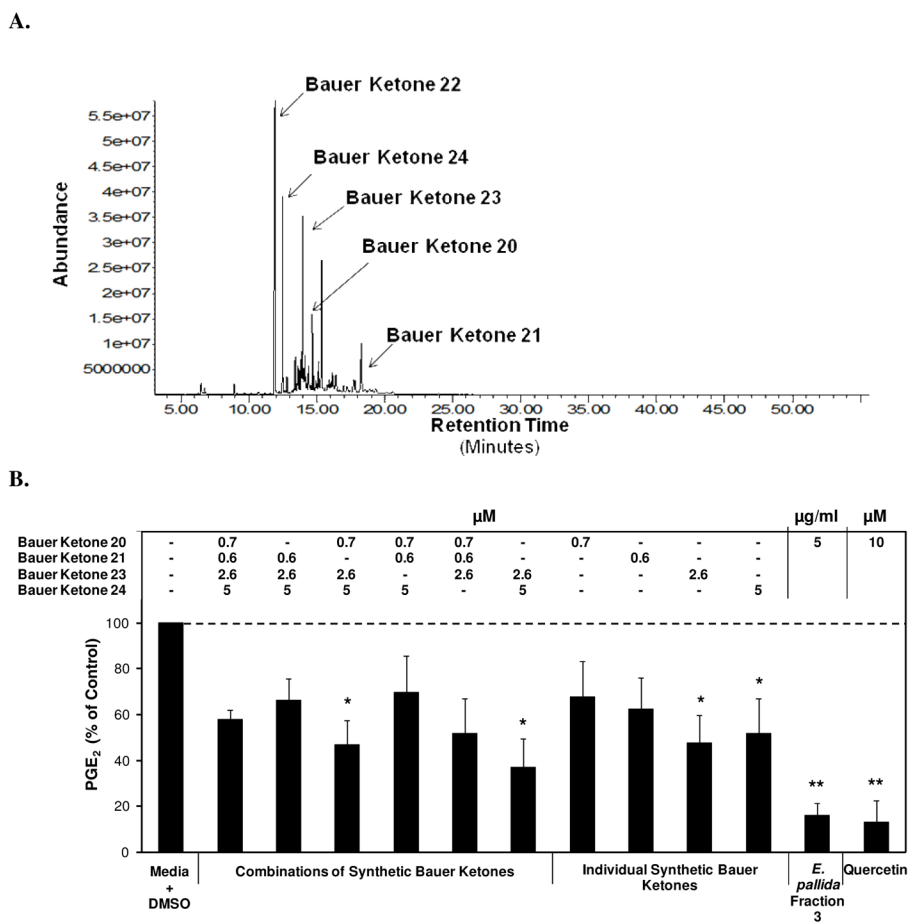


Figure 2.
(A) GC-MS chromatogram of Fraction 3 from *E. pallida*, identifying key ketones. All identified constituents were confirmed via synthetic standards. **(B)** Significant inhibition of LPS induced PGE₂ production in RAW264.7 cells after treatment with chemically synthesized Bauer Ketones at concentrations present in Fraction 3 from *E. pallida*. The black bars represent PGE₂ levels after induction with 1 $\mu\text{g/ml}$ LPS and treatment with an *Echinacea* fraction or synthetic Bauer Ketone ($n = 3$). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % (5.6 ng/ml) with the combination of Bauer Ketones 21, 23, and 24 showing significant reduction of PGE₂ production ($p = 0.032$). Treatments were also performed without LPS induction and compared to the media + DMSO control set at 100 % (0.03 ng/ml) and no significant changes were observed with any of the treatments in this comparison. Media alone did not inhibit PGE₂ production (99 % of control). * and ** are representative of $p < 0.05$ and $p < 0.001$. Each bar represents % of control \pm standard error. Quercetin was used as the positive control.

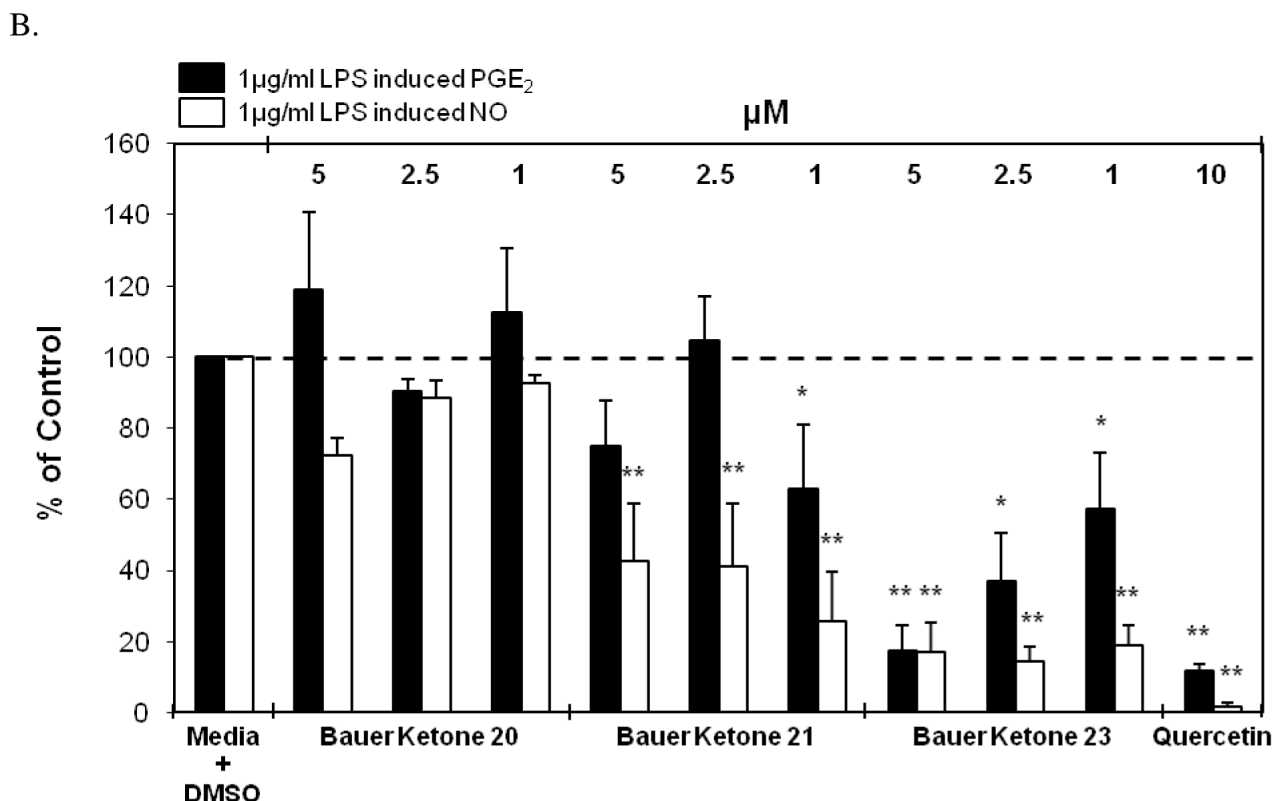
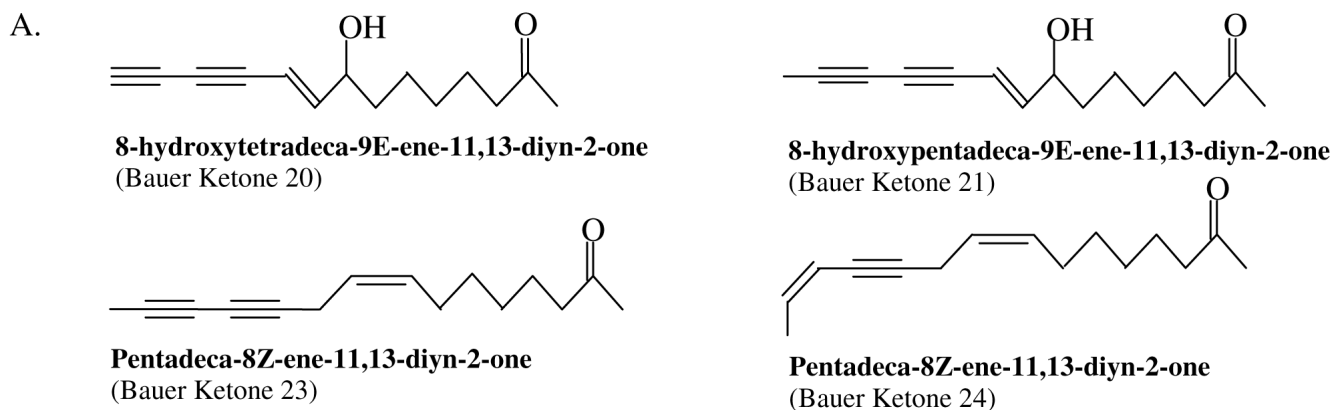


Figure 3.

(A) Structures and nomenclature for Bauer Ketones identified in Fraction 3 from *E. pallida*. (B) Significant inhibition of LPS induced PGE₂ and NO production in RAW264.7 cells after treatment with chemically synthesized Bauer Ketones 21 and 23. The black bars represent PGE₂ levels and the white bars represent NO levels after induction with 1 μg/ml LPS and treatment with a ketone (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (3.7 ng/ml) and NO production (12.4 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ production with Bauer Ketone 21 at 1 μM (p = 0.046). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.02 ng/ml). There was no significant difference in NO production in treatments without LPS. Quercetin was used as a positive control for both studies and showed significant inhibition of PGE₂ and NO production (p < 0.0001). * and ** are representative of p < 0.05 and p < 0.001. Each bar represents

% of control \pm standard error. Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the chemically synthesized Bauer Ketones (data not shown)

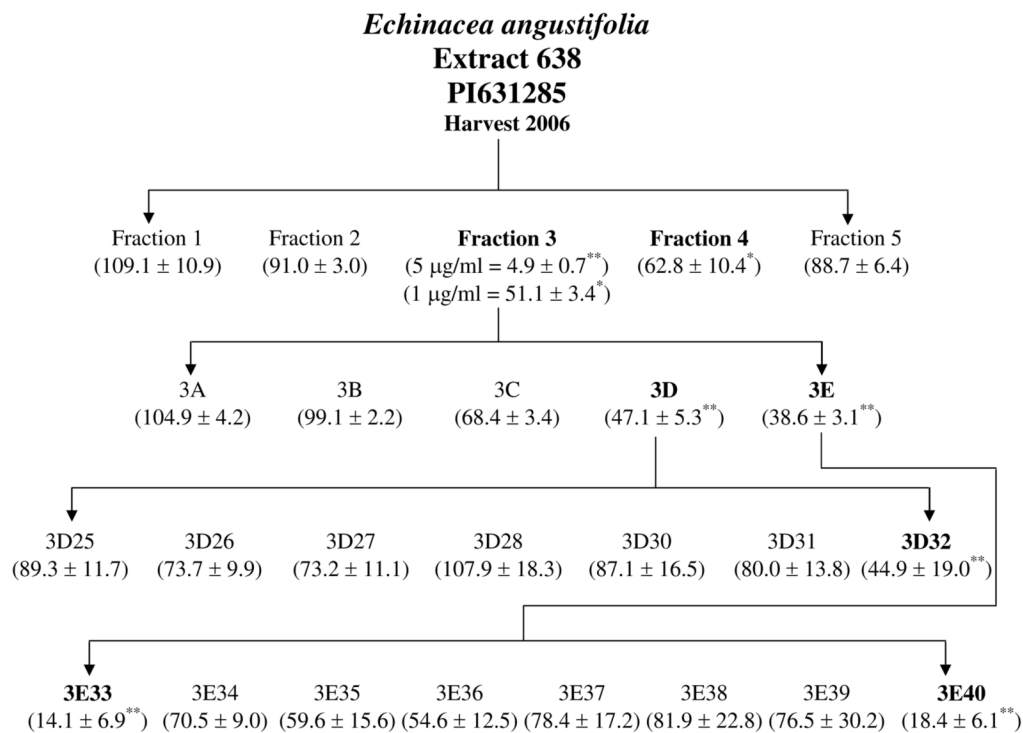


Figure 4. Semi-preparative reverse phased HPLC fractionation scheme of *E. angustifolia* extract from 2006 harvest (PI631285). Bolded fractions represent those showing significant inhibition of LPS induced PGE₂ production. Numbers in parenthesis indicate % of control ± standard error of PGE₂ production compared to the media + DMSO + LPS control set at 100% PGE₂ production. * and ** are representative of p<0.05 and p<0.001. See figures 4–8 for details on PGE₂ data including concentrations studied.

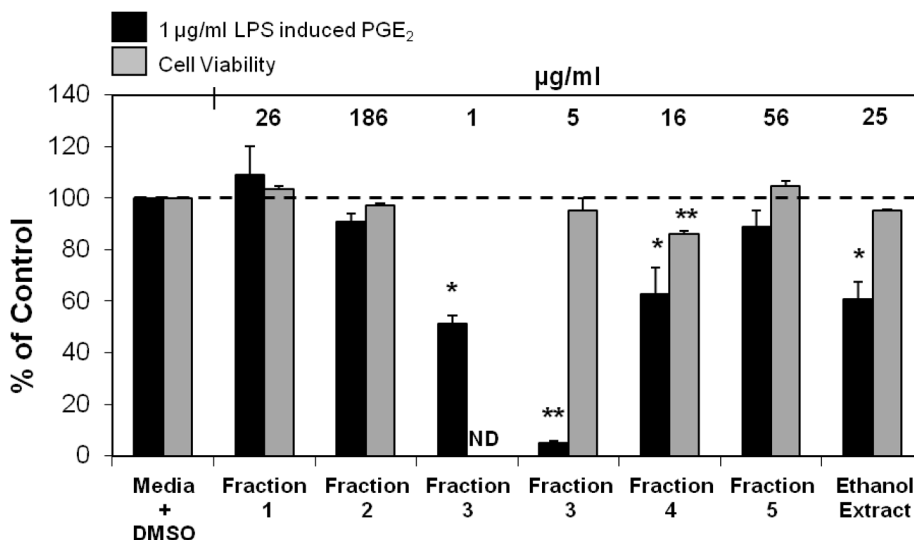


Figure 5.

Inhibition of LPS induced PGE₂ production and cytotoxicity analysis after treatments with first round fractions from a 2006 extract of *E. angustifolia* (PI631285) in RAW264.7 cells. The black bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction or ethanol extract (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (2.2 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ with fractions 1, 3, 4, and ethanol extract (p ≤ 0.035). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.1 ng/ml). Baicalein was used as the positive control in the PGE₂ analysis and showed significant inhibition of PGE₂ production (p < 0.0001). ND indicates analysis not determined. Ursolic acid was used as a positive control in the cytotoxicity assay and showed significant cell death at 30 µM and 50 µM (p < 0.0001). Media alone was also used as a negative control showing no significant inhibition of PGE₂ or cytotoxicity. * and ** are representative of p < 0.05 and p < 0.001. Since Fraction 3 showed no cytotoxicity at 5 µg/ml it was not assessed for cytotoxicity at 1 µg/ml. Each bar represents % of control ± standard error.

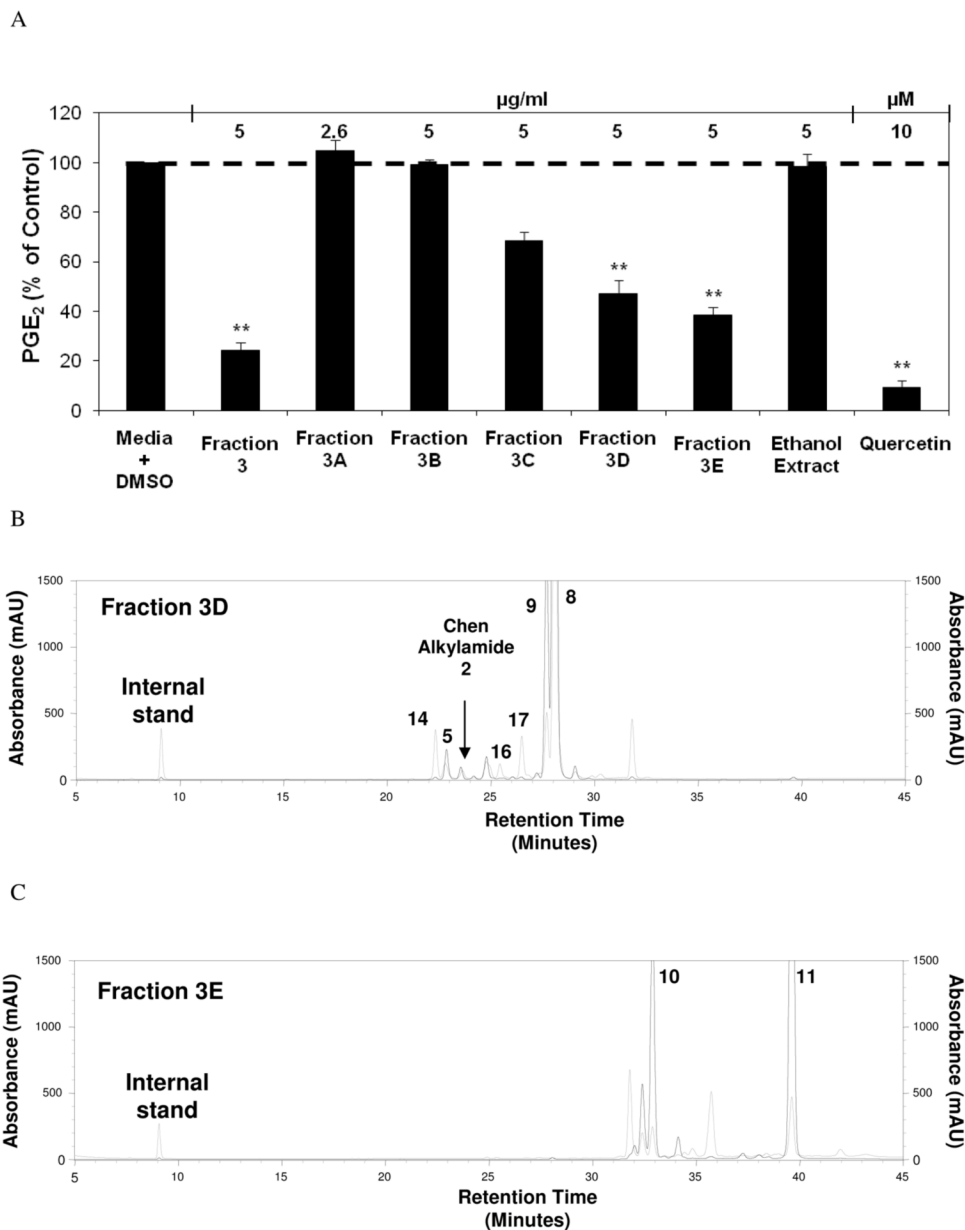


Figure 6. (A) Inhibition of LPS induced PGE₂ production in RAW264.7 cells after treatments with second round fractions from Fraction 3 of *E. angustifolia* (from figure 4). The bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction or ethanol extract (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (1.8 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ with Fraction 3 and 3E (p ≤ 0.029). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.1 ng/ml). Baicalein and quercetin were used as positive controls. Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the fractions (Data not shown). * and ** are representative of p < 0.05 and p < 0.001. Each bar represents % of control ± standard error. (B) HPLC chromatograms of second round Fractions 3D and (C) 3E, identifying key alkylamides (Quantification from HPLC present in Table 2). Black lines

represent 260 nm and grey lines represent 330 nm. The internal standard used for both **(B)** and **(C)** was *N*-isobutylundeca-2-ene-8,10-diynamide ($C_{15}H_{21}O_2$).

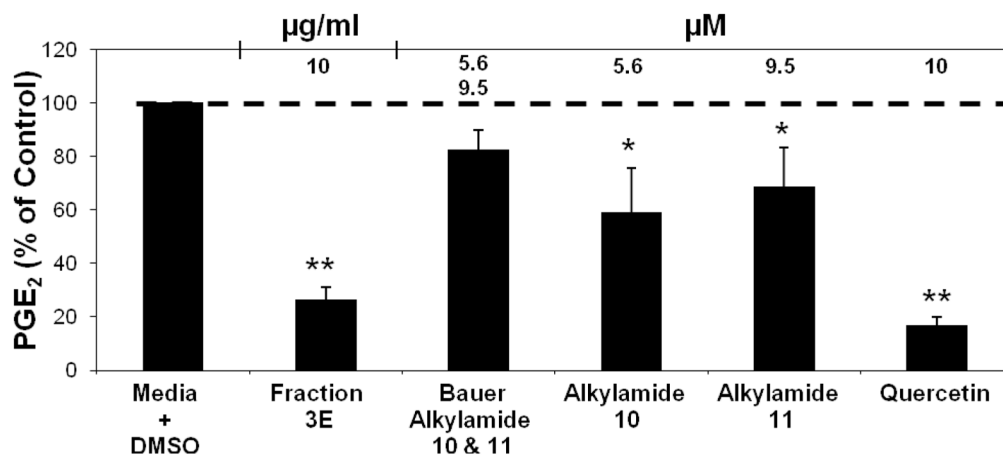
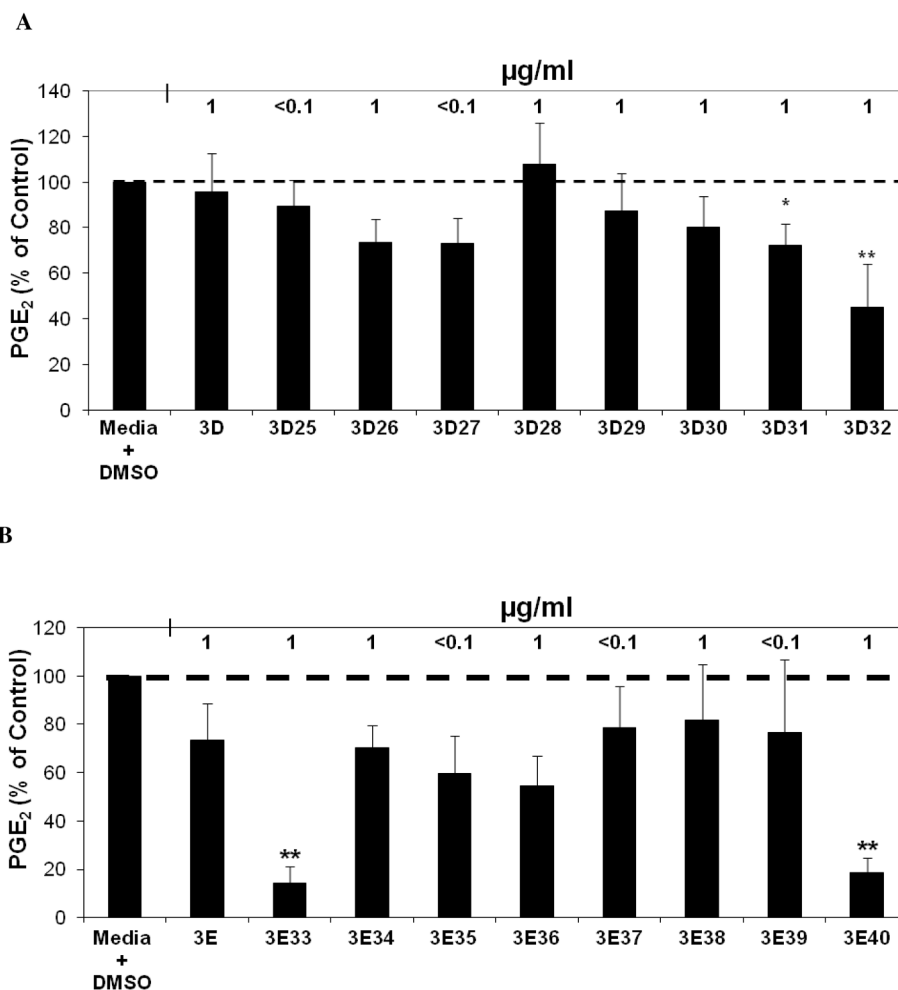


Figure 7. Combination of Bauer Alkylamides 10 and 11 at the concentrations found in fraction 3E (Table 2). The bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (4.4 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ with fraction 3E (p = 0.0275). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.2 ng/ml). Quercetin was used as the positive control. * and ** are representative of p<0.05 and p<0.001. Each bar represents % of control ± standard error.

**Figure 8.**

(A) Inhibition of LPS induced PGE₂ production analysis after treatments with *E. angustifolia* third round D fractions. The black bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction or ethanol extract (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (2.8 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ with Fractions 3D, 3D26, 3D27, 3D30, and 3D31 (p ≤ 0.04). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.2 ng/ml). Baicalein was used as a positive control (p < 0.05). Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the third round fractions. * and ** are representative of p < 0.05 and p < 0.001. Each bar represents % of control ± standard error.

(B) Inhibition of LPS induced PGE₂ production after treatments with *E. angustifolia* third round E fractions. The black bars represent PGE₂ levels after induction with 1 µg/ml LPS and treatment with an *Echinacea* fraction or ethanol extract (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (1.9 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE₂ with fractions 3E, 3E33, 3E34, 3E36, 3E37, and 3E38 (p ≤ 0.027). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE₂ production (0.1 ng/ml). Baicalein was used as a positive control (p < 0.05). Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the third round fractions. * and ** are representative of p < 0.05 and p < 0.001. Each bar represents % of control ± standard error.

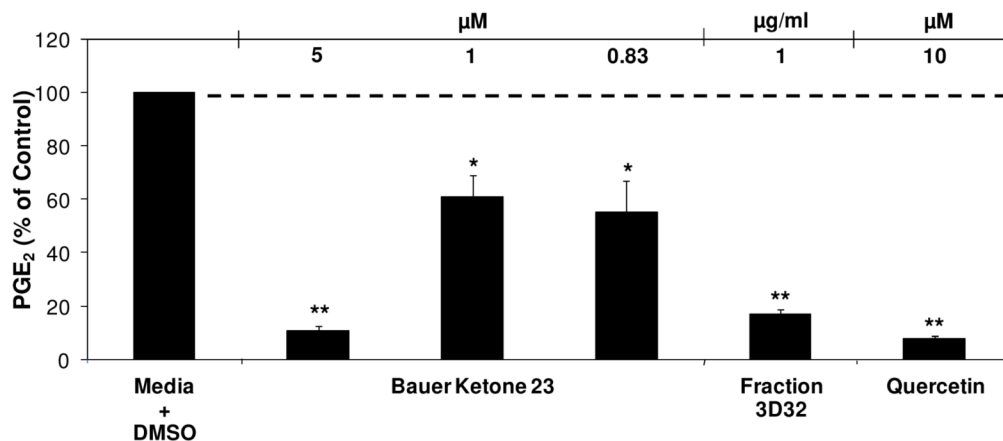


Figure 9.

Inhibition of LPS induced PGE₂ production after treatment of synthetic Bauer Ketone 23 at concentration present in third round *E. angustifolia* Fraction 3D32. The black bars represent PGE₂ levels after induction with 1 μg/ml LPS and with the ketone or fraction (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (3.8 ng/ml). Treatments were also performed without LPS induction showing no significant differences in PGE₂ production. Quercetin was used as a positive control. * and ** are representative of p<0.05 and p<0.001. Each bar represents % of control ± standard error. A parallel cytotoxicity screen was conducted yielding no significant cytotoxicity with Bauer Ketone 23 at the concentrations measured (data not shown).

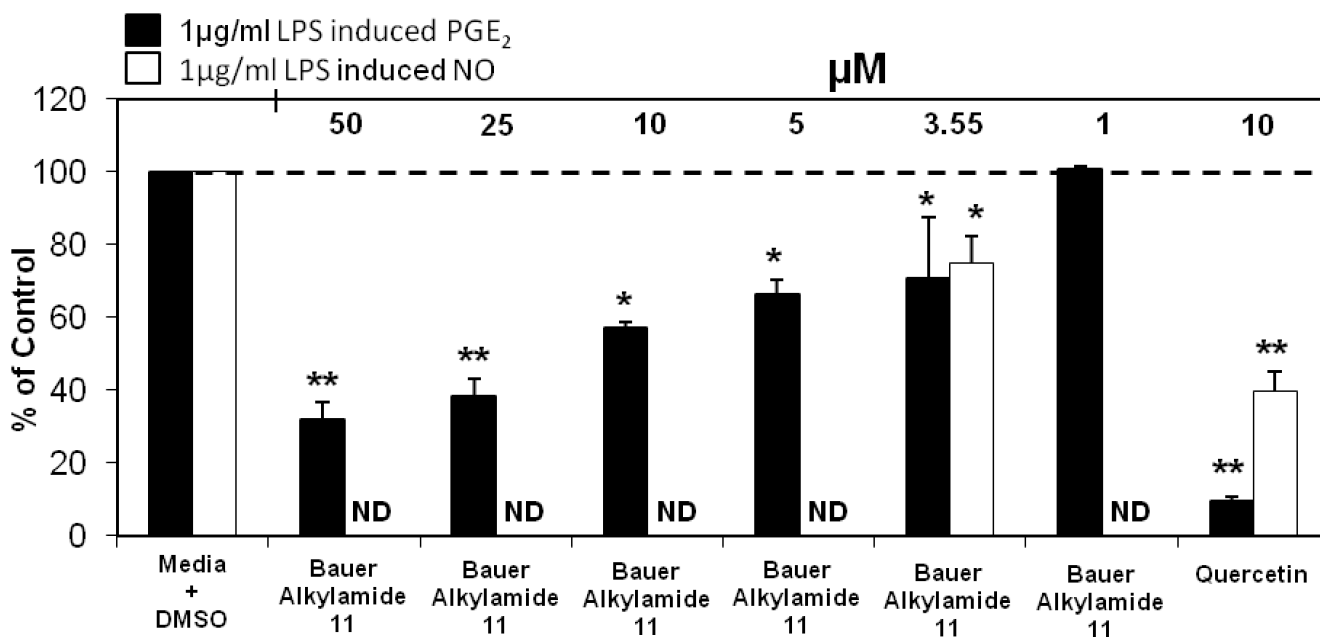


Figure 10.

Significant inhibition of LPS induced PGE₂ and NO production in RAW264.7 cells after treatment with chemically synthesized Bauer Alkylamide 11. The black bars represent PGE₂ levels and the white bars represent NO levels after induction with 1 μg/ml LPS and treatment with alkylamide (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % PGE₂ production (4.7 ng/ml) and NO production (11.6 ng/ml). Treatments were also performed without LPS induction showing no significant differences in PGE₂ or NO production. ND indicates analysis not determined. Quercetin was used as a positive control for both studies and showed significant inhibition of PGE₂ and NO production at 10 μM (p<0.0001). * and ** are representative of p<0.05 and p<0.001. Each bar represents % of control ± standard error. Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with Bauer Alkylamide 11 at the concentrations screened (data not shown).

Table 1

Inhibition of LPS induced PGE₂ production and cytotoxicity analysis after treatments with *E. purpurea* and *E. tennesseensis* fractions^a

Species Fraction	Accession	Anti-inflammatory (PGE ₂)		p-value	Cytotoxicity		p-value
		Concentration (µg/ml)	% of Control ± SE		Concentration (µg/ml)	% Control ± SE	
<i>E. purpurea</i>	Fraction 1 P1631307	274	122. ± 22	0.62	274	101 ± 2	0.81
<i>E. purpurea</i>	Fraction 2 P1631307	89	134 ± 10	0.38	189	98 ± 3	0.49
<i>E. purpurea</i>	Fraction 3 P1631307	75	65 ± 21	0.08	75	105 ± 2	0.23
<i>E. purpurea</i>	Fraction 4 P1631307	41	112 ± 11	0.75	41	98 ± 4	0.56
<i>E. purpurea</i>	Fraction 5 P1631307	95	118 ± 21	0.68	95	96 ± 2	0.33
<i>E. purpurea</i>	Fraction 6 P1631307	96	123 ± 15	0.56	96	101 ± 4	0.87
<i>E. purpurea</i>	Fraction 7 P1631307	23	137 ± 39	0.53	23	94 ± 1	0.14
<i>E. purpurea</i>	Ethanol Extract P1631307	25	48 ± 13	0.02	25	105 ± 4	1.0
<i>E. tennesseensis</i>	Fraction 1 P1631250	271	87 ± 9	0.48	577	94 ± 3	0.11
<i>E. tennesseensis</i>	Fraction 2 P1631250	0.14	109 ± 11	0.72	0.3	96 ± 5	0.30
<i>E. tennesseensis</i>	Fraction 3 P1631250	41	104 ± 13	0.91	41	96 ± 3	0.77
<i>E. tennesseensis</i>	Fraction 3 P1631250	20	6 ± 2	<0.0001			
<i>E. tennesseensis</i>	Fraction 3 P1631250	5	24 ± 10	<0.0001			
<i>E. tennesseensis</i>	Fraction 3 P1631250	8	90 ± 21	0.48			
<i>E. tennesseensis</i>	Fraction 4 P1631250	11	119 ± 4	0.43	17	97 ± 3	0.38
<i>E. tennesseensis</i>	Fraction 5 P1631250	25	128 ± 14	0.28	23	89 ± 3	0.01
<i>E. tennesseensis</i>	Ethanol Extract P1631250		89 ± 9	0.56	59	89 ± 1	0.01

^a HPLC was used to fractionate a 2005 extract of *E. purpurea* (P1631370) and a 2005 extract of *E. tennesseensis* (P1631250), yielding no significant inhibition of PGE₂ production at concentrations lower than 20 µg/ml. All treatments were compared to media + DMSO control that was set at 100% for both PGE₂ analysis and cytotoxicity. For *E. purpurea* and *E. tennesseensis*, 100% of control for PGE₂ was 1.7 ng/ml and 2.8 ng/ml, respectively. Baicalein (6 µM) was the positive control for the PGE₂ assay and ursolic acid (10 µM, 30 µM, 50 µM) was the positive control for the MTS cytotoxicity assay. Each fraction or extract represents three replicates ± standard error for both the PGE₂ and cytotoxicity analyses. Cytotoxicity was not performed on dilutions of fractions when the higher concentrations were not cytotoxic.

Table 2HPLC analysis of the constituents found in *E. angustifolia* Fractions 3D and 3E^a

Metabolite	Fraction 3D Metabolite Concentration		Fraction 3E Metabolite Concentration	
	($\mu\text{g/ml}$)	(μM)	($\mu\text{g/ml}$)	(μM)
Chen Alkylamide 2	0.7	0.2	0.0	0.0
Bauer Alkylamide 5	2.1	0.5	0.0	0.0
Bauer Alkylamide 8	36.6	9.0	0.0	0.0
Bauer Alkylamide 9	15.3	3.8	0.0	0.0
Bauer Alkylamide 10	0.0	0.0	22.6	5.6
Bauer Alkylamide 11	0.1	0.03	37.8	9.5
Bauer Alkylamide 14	2.1	0.5	0.0	0.0
Bauer Alkylamide 16	0.8	0.2	0.0	0.0
Bauer Alkylamide 17	1.7	0.5	0.0	0.0

^aHPLC analysis identified alkylamides present in 10 $\mu\text{g/ml}$ of second round fractions 3D and 3E, as well as the metabolites concentration in μM . Structures of alkylamides have been previously described (2,6).

Table 3GC-MS analysis of selected third round *E. angustifolia* fractions^a

<i>Echinacea angustifolia</i> Fraction	Compound Identified	% of Fraction by Dry Weight	Approximate Concentration of Compound (µg)	Approximate Concentration of Compound (µM)
3D28	Bauer Alkylamide 8-9	18	0.18	0.64
3D32	Bauer Ketone 23	75	0.75	0.83
3E33	Bauer Alkylamide 10	34	0.34	0.15
	Bauer Ketone 23	12	0.12	0.25
3E38	Bauer Alkylamide 11	87	0.87	3.47
3E40	Bauer Alkylamide 11	96	0.96	3.55

^aGC-MS analysis identified constituents present in 1 µg/ml of third round fractions, as well as the metabolites concentration. All identified constituents were confirmed via synthetic standards.

Table 4Change in PGE₂ activity over storage time with third round *E. angustifolia* fractions

<i>E. angustifolia</i> Fraction	PGE ₂ production (% of control) August 2007	PGE ₂ production (% of control) June 2008
3E33	14.1 ± 6.9**	133.1 ± 43.5
3E40	18.4 ± 6.1**	81.0 ± 3.9

** indicates significant p-value <0.0001. Bauer Alkylamides and Ketones identified to be present in Table 3 were quantified prior to PGE₂ analysis in 2007 and again after PGE₂ analysis in 2008 yielding no difference in these constituent concentrations. Data represents % of control ± standard error, with 100 % of control for 2007 Fractions 3E33 and 3E40 at 1.9 ng/ml and for 2007 Fraction 3E33 and 3E40 at 3.5 ng/ml and 4.7 ng/ml, respectively.