

Enrichment of *Echinacea angustifolia* with Bauer Alkylamide 11 and Bauer Ketone 23 Increased Anti-inflammatory Potential through Interference with COX-2 Enzyme Activity

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Bauer alkylamide 11 and Bauer ketone 23 were previously found to be partially responsible for *Echinacea angustifolia* anti-inflammatory properties. This study further tested their importance using the inhibition of prostaglandin E₂ (PGE₂) and nitric oxide (NO) production by RAW264.7 mouse macrophages in the absence and presence of lipopolysaccharide (LPS) and *E. angustifolia* extracts, phytochemical enriched fractions, or pure synthesized standards. Molecular targets were probed using microarray, qRT-PCR, Western blot, and enzyme assays. Fractions with these phytochemicals were more potent inhibitors of LPS-induced PGE₂ production than *E. angustifolia* extracts. Microarray did not detect changes in transcripts with phytochemical treatments; however, qRT-PCR showed a decrease in TNF- α and an increase of iNOS transcripts. LPS-induced COX-2 protein was increased by an *E. angustifolia* fraction containing Bauer ketone 23 and by pure phytochemical. COX-2 activity was decreased with all treatments. The phytochemical inhibition of PGE₂ production by *Echinacea* may be due to the direct targeting of COX-2 enzyme.

KEYWORDS: *Echinacea angustifolia*; prostaglandin E₂; nitric oxide; Bauer alkylamides; Bauer ketones; anti-inflammatory; fractionation

INTRODUCTION

Echinacea has been used medicinally for hundreds of years for the treatment of numerous ailments, including inflammation (1), and for the stimulation of the immune system (2). Several studies have been conducted to elucidate the cellular mechanism of action for the immune modulatory properties of *Echinacea* (3–6). These studies have mainly focused on one particular class of compounds of *Echinacea*, alkylamides, and their ability to interact with cannabinoid receptors.

The cannabinoid receptors, CB1 and CB2, are G protein coupled receptors that have been implicated in the modulation of the central nervous system and the inflammatory response. CB1 receptors occur in neurons from the central and peripheral nervous system and are concentrated in the brain, whereas CB2 receptors occur mainly in immune cells, including macrophages (7). Studies attempting to unravel the mechanism of action for the immune modulatory effects of *Echinacea* have led to the finding that alkylamides, which are a class of constituents prominent in certain *Echinacea* species, can act as cannabinomimetics (8). Endogenous ligands for the cannabinoid receptors including anandamide (AEA) and 2-arachidonyl glycerol (2-AG) share structural similarity with

Echinacea alkylamides (9). Previous studies have determined that certain alkylamides have the ability to bind to the CB2 receptor with K_i values around 60 nM with greater affinity than the natural ligands (8). The evidence for CB2 receptor involvement in the immune modulatory effect of *Echinacea* was further strengthened by Gertsch et al., who provided evidence that the gene expression of an important inflammatory cytokine released by macrophage cells, tumor necrosis factor alpha (TNF- α), was induced via the CB2 receptor by a commercial preparation of *Echinacea* called Echinaforce (4). This activity was attributed to certain alkylamides present in the Echinaforce preparation, including Bauer alkylamide 11 at 0.5 μ M (the Bauer numbers are from ref 10). Recently, *Echinacea* ketones have also been shown to have antitumorigenic and anti-inflammatory properties in human cancer cells and mouse macrophage cells, respectively (11, 12). Therefore, Egger et al. set out to determine whether various ketoalkenes of *Echinacea pallida* could mediate immune modulatory effects through the cannabinoid receptors; using a steady-state GTPase assay to assess cannabinoid receptor antagonistic activity, they identified no significant activity with the ketoalkenes from *E. pallida* (3).

Significant inhibition of prostaglandin E₂ (PGE₂) and nitric oxide (NO) production have been achievable with treatments of *Echinacea* extracts, purified fractions, and pure constituents providing two excellent end points for the elucidation of species,

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as well as classes of compounds, that are important for the in vitro anti-inflammatory properties of *Echinacea* (12–15). Upstream enzymes of these end points, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), for PGE₂ and NO, respectively, have been studied to further delineate how *Echinacea* modulates inflammation. Muller-Jakic et al. determined that alkylamides isolated from a Soxhlet *n*-hexane extract of *Echinacea angustifolia* were capable of inhibiting both cyclooxygenase and 5-lipoxygenase activity in vitro (16). Another study showed that certain alkylamides from a CO₂ extract of *E. angustifolia* abrogated COX-2 activity, but had no effect on COX-2 mRNA or protein in neuroglioma cells (17). Zhai et al. described the inhibition of NO identified with Soxhlet ethanol extracts of *E. angustifolia*, *E. pallida*, and *Echinacea purpurea* as being due to an inhibition of iNOS protein expression, attributing this effect to the lipophilic alkylamides (15).

The presence of Bauer alkylamide 11 at a concentration of 3.55 μ M and Bauer ketone 23 at a concentration of 0.83 μ M in *E. angustifolia* fractions capable of inhibiting PGE₂ production was a key finding in our laboratory (12). During a refractionation of *E. angustifolia* we identified a fraction capable of significant PGE₂ inhibition, but the potency of this inhibitory activity was less than previously identified in our prior studies. We determined that the decreased inhibitory activity may be due to the lower concentration of Bauer alkylamide 11 and the absence of Bauer ketone 23 in the new fraction. This observation led to the hypothesis that these two constituents at the proper concentration ratios in *E. angustifolia* fractions may target specific bioactivities. Our studies were conducted to understand the anti-inflammatory roles, via inhibition of PGE₂ and NO production, of Bauer alkylamide 11 and Bauer ketone 23 in an *E. angustifolia* fraction and elucidate a mechanism of action leading to the modulation of these inflammatory end points in the RAW264.7 mouse macrophage cell line.

MATERIALS AND METHODS

Plant Material and Extraction. *E. angustifolia* (PI636395) root material from a 2008 harvest was grown in Ames, IA, by the USDA North Central Regional Plant Introduction Station (NCRPIS, Ames, IA) and collected, stored, and Soxhlet extracted with 95% ethanol as previously described (13). Further information about the accessions and requests for the germplasm can be found on the Germplasm Resources Information Network database at http://www.ars-grin.gov/npgs/acc/acc_queries.html provided by NCRPIS.

Semipreparative HPLC Fractionation. The fractionation of a 95% ethanol extract of *E. angustifolia* was performed using semipreparative reversed-phase HPLC with methods to avoid endotoxin contamination as previously described (12). Five fractions were produced from the fractionation, with the alkylamide-rich fraction 3 responsible for the majority of identified bioactivity described under Results. The activity associated with fraction 3 from *E. angustifolia* in the present study was consistent with our previous work (10, 11).

GC-MS Analysis. GC-MS analyses was used to determine concentrations of known compounds present in *E. angustifolia* fractions through the use of synthetic standards as described by LaLone et al. (12).

Alkylamide and Ketone Synthesis. Alkylamide and ketone synthesis was conducted as described previously, accounting for percent purity when determining concentrations of compounds (12, 13).

Cell Culture. RAW264.7 mouse monocyte/macrophage cells were purchased from American Type Culture Collection (catalog: TIB-71; Manassas, VA). The conditions under which the cells were cultured have been previously described (13, 18).

***E. angustifolia* Fraction and Constituent Treatments.** Five treatments were consistently used for each assay: (1) *E. angustifolia* fraction 3 at 1 μ g/mL; (2) enriched fraction 3 consisting of *E. angustifolia* fraction 3 at 1 μ g/mL (which contained Bauer alkylamide 11 at a concentration of 0.05 μ M), synthetic Bauer alkylamide 11 at 3.5 μ M, and Bauer ketone 23 at

0.83 μ M; (3) the combination of Bauer alkylamide 11 at 3.55 μ M and Bauer ketone 23 at 0.83 μ M; (4) chemically synthesized Bauer alkylamide 11 at 3.55 μ M; and (5) chemically synthesized Bauer ketone 23 at 0.83 μ M.

Measurement of PGE₂, NO, and Cytotoxicity. The production of PGE₂ was assessed using a PGE₂ enzyme immunoassay (GE Biosciences, Piscataway, NJ) after treatment of RAW264.7 mouse macrophage cells for 8 h with fractions from *E. angustifolia* and with or without lipopolysaccharide (*Escherichia coli* O26:B6, Sigma, St. Louis, MO) as previously described (13). Quercetin (3,5,7,3',4'-pentahydroxyflavon) was chosen as the positive control for this assay due to its anti-inflammatory properties at a concentration of 10 μ M (Sigma).

NO production was analyzed after a 24 h incubation with *Echinacea* fraction or pure constituent using Griess Reagent System (Promega, Madison, WI) following the manufacturer's protocol. The assay has been previously described using the RAW264.7 cell line and outlined in LaLone et al. (12).

Cytotoxicity was analyzed using the Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega) as previously described (13). All fractions and compounds were screened for cytotoxicity at the concentrations in the PGE₂ assay and incubated for 24 h.

Western Blots. RAW264.7 cells were grown in 10 cm Petri dishes to 80% confluency (overnight) and treated for 8 h. Cells were washed twice with ice-cold 1 \times phosphate-buffered saline (PBS) and lysed on ice for 5 min with 500 μ L of lysis buffer (50 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol tetraacetic acid, 150 mM sodium chloride, 2 mM phenylmethanesulfonyl fluoride, 25 mM leupeptin, 10 mM aprotinin, 10 mM sodium fluoride, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5% Triton X-100). After lysis, the cells were collected using a rubber policeman and centrifuged at 4 $^{\circ}$ C to form a cell pellet. The supernatant was isolated, and the protein concentration in the cell lysate was identified using the BCA Protein Assay Reagent (Pierce, Rockford, IL). COX-1 (sc-19998), COX-2 (sc-19999), iNOS (sc-7271), and α -tubulin (sc-8035) mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:2000, 1:1000, 1:600, and 1:2000, respectively, in 5% milk Tris-buffered saline containing 0.5% Tween-20. Immunoblot separation was carried out as described by Przybyszewski et al. (19) and visualized using ECL detection. Quantity One software was used for semiquantitative analysis as previously described (18).

RNA Extraction and DNase Digestion. RAW264.7 mouse macrophage cells were grown in 75 cm flasks to 80% confluency and treated for 8 h for the microarray study. The cells were grown in 6-well plates to 80% confluency for the qRT-PCR studies collected at six separate time points (0.5, 1, 2, 4, 8, and 24 h). The treatments selected for the qRT-PCR studies were *E. angustifolia* fraction 3, enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually. These treatments were incubated with and without LPS. Cells were collected using a rubber policeman after the flask or plate had been washed twice with PBS. The Trizol method (Invitrogen, Carlsbad, CA) of extraction was used to isolate RNA, and further purification was carried out using an RNeasy purification kit in combination with an RNase-free DNase kit (Qiagen, Valencia, CA). Following extraction, RNA was analyzed for quality and quantity using an Agilent Bioanalyzer 2100 and RNA Nano 6000 Labchip kit (Agilent Technologies, Palo Alto, CA).

Microarray. The microarray experiment was carried out using the GeneChip Mouse Genome 430A 2.0 Array, which consisted of approximately 22600 probe sets, representing 14500 well-substantiated genes (Affymetrix, Santa Clara, CA). The five treatments selected for the microarray analysis were the medium + DMSO and medium + DMSO + LPS controls and *E. angustifolia* fraction 3 (1 μ g/mL) + LPS, fraction 3 (1 μ g/mL) + synthetic Bauer alkylamide 11 (3.5 μ M) + Bauer ketone 23 (0.83 μ M) + LPS, and synthetic Bauer alkylamide 11 (3.55 μ M) + Bauer ketone 23 (0.83 μ M) + LPS. The controls were used to establish that the LPS effect was consistent with current literature on microarray studies with RAW264.7 macrophages. Our study was to determine differentially expressed (DE) genes important for the inhibition of LPS-induced PGE₂ production; therefore, treatments without LPS were not included in the microarray study. Four replicates of each treatment were analyzed on separate chips, and RNA labeling was performed according to the manufacturer's recommendations (Affymetrix). The gene chips were run using a Gene Chip fluidics station 450 and a GeneChip Scanner 3000 7G conducted at the Gene Chip Facility at Iowa State University.

qRT-PCR. An iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to reverse-transcribe RNA to cDNA. Primers were designed with an annealing temperature of 55 °C for COX-1, COX-2, TNF- α , iNOS, and GAPDH using Primer3 (20) and ordered from Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). Amplification conditions for the qRT-PCR were set at 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min and 55 °C for 1 min.

Plasmid preparation for standard curves has been described previously (21), with the exception that PCR products were cloned into MAX Efficiency DH5 α Competent Cells.

COX Activity Assay. COX activity was measured using a COX Fluorescent Activity Assay Kit according to the manufacturer's directions

Table 1. Primers Used for qRT-PCR Analysis

gene	primer	oligonucleotide sequence (5'–3')
COX-1	S	CCTCACCAGTCAATCCCTGT
	AS	GTAGCCCGTGCGAGTACAAT
COX-2	S	TTGGGGAGACCATGGTAGAG
	AS	GCTCGGCTCCAGTATTGAG
TNF- α	S	AGGAGGGAGAACAGAAACTC
	AS	AATGAGAAGAGGCTGAGACA
iNOS	S	GTCTTGGTAAAGTGGTGT
	AS	GTGCTTGCCCTTACTGCTC
GAPDH	S	CAATGTGTCGCTCGTGGAT
	AS	AGCCCAAGATGCCCTTCAG

(Cayman Chemicals, Ann Arbor, MI) after an 8 h treatment with *E. angustifolia* fraction 3, enriched fraction 3, and the combination of Bauer alkylamide 11 and Bauer ketone 23, as well as these constituents individually (with and without LPS). The assay measures the peroxidase component of the cyclooxygenase enzymes by monitoring the reaction between PGG₂ and 10-acetyl-3,7-dihydroxyphenoxazine after the addition of arachidonic acid, which generates the fluorescent compound resorufin. The common plant flavonoid, quercetin, was used as a positive control at 25 μ M.

Milliplex. RAW264.7 cells were plated in 24-well plates (1.57×10^5 cells/well) and grown overnight. The treatments that were selected for analysis were the *E. angustifolia* fraction 3, enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually. Each treatment was applied with and without LPS induction (1 μ g/mL) and treated for 24 h. After the 24 h treatment, the supernatant was collected for the assay. A 32-plex Millipore cytokine/chemokine kit was used to analyze cell supernatants on a multiplex assay system (Bioplex, Bio-Rad).

Statistical Analysis. Both log transformed PGE₂ data and NO data and COX activity data were analyzed using randomized complete block design with several levels of treatment, followed by a *t* test based on a pooled error variance to determine statistical significance compared to the (medium + DMSO + LPS) control. In all figures, the data are represented as percent of control \pm standard error, normalizing the (medium + 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 percent of control \pm standard error, normalizing the (medium + DMSO) control to 100% cell survival. The qRT-PCR data

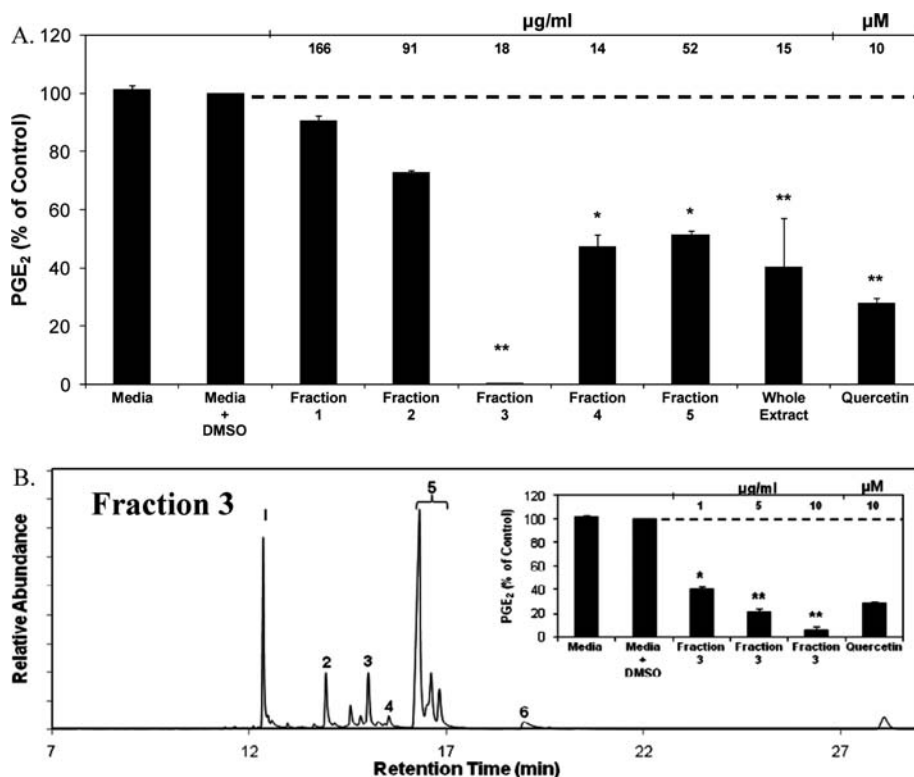


Figure 1. (A) Fraction 3 from a 2009 extract of *Echinacea angustifolia* (PI631293) significantly inhibited PGE₂ production in RAW264.7 cells. The black bars represent PGE₂ levels after induction with 1 μ g/mL LPS and treatment with an *Echinacea* fraction or ethanol extract ($N = 3$). All treatments + LPS were compared to medium + DMSO + LPS control that was set at 100% (4.3 ng/mL). Treatments without LPS were compared to the medium + DMSO control set at 100% (0.03 ng/mL). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. Each bar represents percent of control \pm standard error. Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the fractions or the extract (data not shown). (B) Gas chromatography analysis of bioactive fraction 3 from *E. angustifolia*. Total ion chromatograms of fraction 3 with peaks for which chemical identity was established by comparing their retention times and mass spectra to authentic standards: Bauer alkylamide 13 (1), Bauer alkylamide 12 (2), Bauer alkylamide 10 (3), Bauer alkylamide 11 (4), Bauer alkylamide 8/9 (5), and Bauer alkylamide 14 (6). Quantification of Bauer alkylamide 11 yielded a concentration of 0.05 μ M.

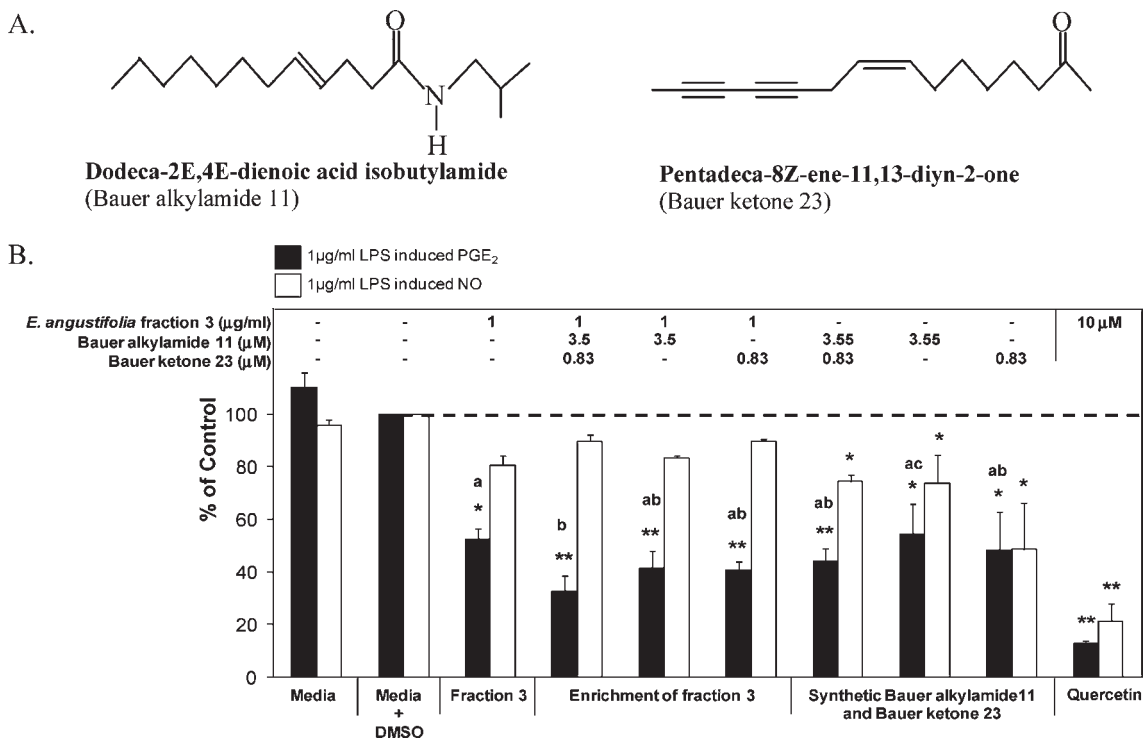


Figure 2. (A) Structures and nomenclature for Bauer alkylamide 11 and Bauer ketone 23. (B) LPS-induced PGE₂ and NO production in RAW264.7 cells treated with *E. angustifolia* fraction 3, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and Bauer ketone 23. The black bars represent PGE₂ levels, and the white bars represent NO levels after induction with 1 μg/mL LPS and treatment ($N = 3$). All treatments + LPS were compared to medium + DMSO + LPS control that was set at 100% PGE₂ production (2.5 ng/mL) and NO production (18.8 ng/mL). The treatments without LPS were compared to the medium + DMSO control set at 100% PGE₂ production (0.05 ng/mL) and NO production (~0 ng/mL), identifying no significant differences with treatment for either end point. 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 $p < 0.001$, respectively. Each bar represents percent of control \pm standard error. Parallel cytotoxicity screens were conducted yielding no significant cytotoxicity with any of the treatments or combination of treatments (data not shown).

were analyzed using two-way ANOVA for the log starting quantity with treatment and time as factors. Each treatment was compared to the medium + DMSO + LPS control. Western blot analysis was previously described (22). All statistical analysis was conducted using the GLM procedures in SAS (version 9.1, SAS Institute Inc., Cary, NC).

The microarray experiment had a randomized complete block design with four replications as fixed block and five treatments (medium + DMSO, medium + DMSO + LPS, fraction 3 + LPS, enriched fraction 3 + LPS, Bauer alkylamide 11 + Bauer ketone 23 + LPS). Within each replication, the cultured cells and gene chips were randomly assigned to receive one of the five treatments. The raw data were normalized by the robust multiarray average method (RMA) using the *affy* package in Bioconductor 2.0.8. The log expression data were then analyzed with SAS version 9.1.

All of the pairwise comparisons of interest were tested by unadjusted t test. Differentially expressed genes were identified with a false discovery rate (FDR) of $<0.001\%$ using Benjamini and Hochberg's method (23).

Genes with significant treatment effects (FDR $< 0.001\%$) were included in a hierarchical cluster analysis. The standardized averages for each treatment of each gene were used to compute a Euclidean distance matrix. The average linkage method was used to measure the distance between clusters. The analysis was done in R version 2.5.1.

RESULTS

Enrichment of *E. angustifolia* Fraction. Semipreparative reversed phase HPLC was used to fractionate an extract of root material from *E. angustifolia* into five fractions, and these fractions were analyzed for their effect on LPS-induced PGE₂ production in the RAW264.7 mouse macrophage cell line. The study led to the identification of three fractions that were capable of significantly inhibiting PGE₂ production (Figure 1A). Fraction 3, an alkylamide-rich

fraction equivalent in preparation to the *E. angustifolia* fraction 3 (inhibited to 1.1 ng/mL PGE₂) published by LaLone et al. (10), showed potent PGE₂ reduction compared to medium + DMSO control (1.8 ng/mL PGE₂) at a concentration as low as 1 μg/mL (Figure 1B). GC-MS analysis was conducted to identify the prominent constituents present in fraction 3, as well as to quantify the concentrations of Bauer alkylamide 11 and Bauer ketone 23 (Figure 1B). From this analysis it was determined that Bauer alkylamide 11 (Figure 2A) was present at a concentration of 0.05 μM and there was no trace of Bauer ketone 23 (Figure 2A) in 1 μg/mL of fraction 3. Previously published studies conducted by our laboratory found that Bauer alkylamide 11 and Bauer ketone 23, at concentrations of 3.55 and 0.83 μM, respectively, contributed to the inhibition of PGE₂ production. Therefore, a 3.5 μM concentration of chemically synthesized Bauer alkylamide 11 and a 0.83 μM concentration of Bauer ketone 23 were added to 1 μg/mL of fraction 3 to produce an enriched fraction 3. *E. angustifolia* fraction 3, enriched fraction 3, and combinations of individual constituents and fraction 3 were evaluated after an 8 h treatment with the RAW264.7 cells for PGE₂ and NO production, showing significant PGE₂ inhibition with all treatments and significant NO inhibition with the combination of chemically synthesized Bauer alkylamide 11 and Bauer ketone 23 and each constituent individually (Figure 2B). The enriched fraction 3 was found to have significantly greater PGE₂ inhibition capabilities than the fraction by itself. Cytotoxic effects were not identified with any of the five *E. angustifolia* fractions or with the enriched fraction or combinations of fraction and constituents (data not shown).

Gene expression was analyzed with GeneChip Mouse Genome 430A 2.0 Arrays to identify target genes and determine pathways

leading to the inhibition of PGE₂ production in the RAW264.7 mouse macrophage cells with selected treatments from **Figure 2B** treated for 8 h. The medium + DMSO control was compared to the medium + DMSO + LPS control to establish that the expected genes were differentially expressed. With a FDR of 0.001% 3354 differentially expressed (DE) genes were identified between the controls, which corresponded with the DE genes identified by Hammer et al. (21). Of these 3257 DE genes, 1253 had increased expression levels (5.5% of total probe sets) and 2004 (8.9% of total probe sets) had decreased expression levels, with 731 genes decreased at least 50% below the expression level of the medium + DMSO control and 951 genes increased at least 50% above the expression level of medium + DMSO control after LPS treatment, which represented 3.2 and 4.2% of the total probe sets, respectively. Genes that were increased by the LPS treatment were involved in the inflammatory response, cell cycle, cell signaling, and cell proliferation, and those genes that were decreased were involved in immune response, cell death, and cell motility.

In the search for DE genes between the medium + DMSO + LPS control, *E. angustifolia* fraction 3 + LPS, enriched fraction 3 + LPS, and the combination of Bauer alkylamide 11 + Bauer ketone 23 + LPS, no genes were identified with a FDR below 50%. The eight DE genes that were identified between LPS-treated samples with FDRs between 50 and 75% were analyzed by MetaOmGraph (24) indicating that none were genuine DE genes. **Figure 3A** provides a visual representation of the general gene expression pattern with all five treatments through the use of hierarchical clustering. Two clusters can be identified, one containing the medium + DMSO control and the other consisting of all samples treated with LPS. **Figure 3B** represents changes in gene expression level with the different treatment groups, consistent with the analysis showing the LPS effect with the controls, without any differences between each treatment + LPS.

To obtain a more thorough understanding of the effect of *E. angustifolia* fraction 3, enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually on gene expression, qRT-PCR was used to produce a time course for genes expected to be modulated by *Echinacea*. Genes chosen for this analysis included COX-1, COX-2, TNF- α , iNOS, and GADPH. These studies indicated that COX-2 gene expression is not significantly different throughout the time course from 0.5 to 24 h (**Figure 4B**). The only significant increase in COX-2 gene expression was with the LPS-treated samples compared to the medium + DMSO control. TNF- α gene expression was significantly increased with treatment of the RAW264.7 cells with Bauer ketone 23 at the 0.5 h time point compared to the medium + DMSO + LPS control (**Figure 4C,E**). At the 1 h time point TNF- α gene expression was significantly decreased with treatment of Bauer alkylamide 11 (**Figure 4C,E**). When gene expression analysis for TNF- α was carried out to 24 h, a significant decrease in gene expression was identified for *E. angustifolia* fraction 3 + LPS, enriched fraction 3 + LPS, and combination of Bauer alkylamide 11 and Bauer ketone 23 + LPS as well as with each individual constituent + LPS compared to the medium + DMSO + LPS control (**Figure 4C,E**). Treatments were also carried out without LPS showing no significant difference in TNF- α gene expression compared to the medium + DMSO control (data not shown). LPS-induced TNF- α protein production was also analyzed, indicating that each treatment + LPS, except for the synthetic Bauer ketone 23, was capable of significant inhibition (**Figure 5**). A significant difference in iNOS gene expression was identified at the 24 h time point, indicating an increase in iNOS mRNA with the enriched fraction 3 compared to the medium + DMSO + LPS control (**Figure 4D,F**). The expected LPS induction of the RAW264.7 macrophage cells was identified with the

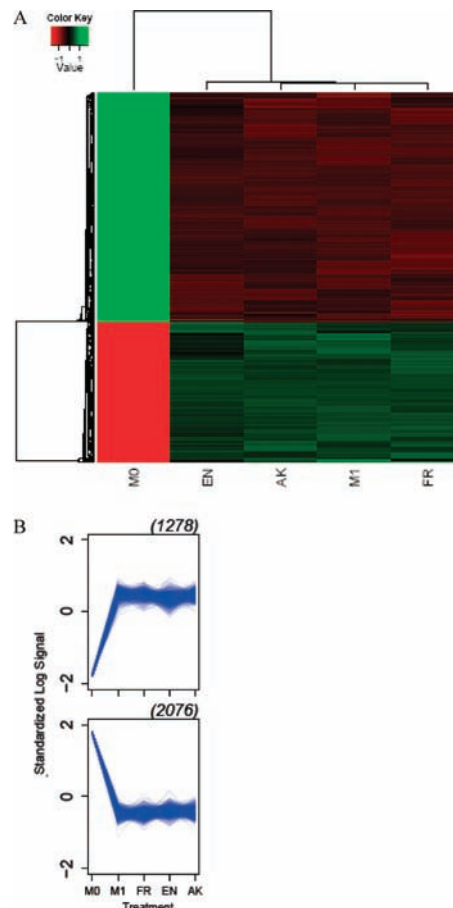


Figure 3. (A) Hierarchical cluster analysis of differentially expressed genes in RAW264.7 mouse macrophages. A total of 3354 differentially expressed probe sets were identified comparing the medium + DMSO control to the medium + DMSO + LPS control with a FDR of 0.001%, with no differentially expressed genes identified between treatments with LPS. On the heatmap, the rows represent the genes and the columns represent the treatments. The red color is indicative of low gene expression, and green is indicative of high gene expression. Treatments are labeled as follows: M0 = medium + DMSO control, M1 = medium + DMSO + LPS control, FR = *E. angustifolia* fraction 3, EN = *E. angustifolia* fraction 3 enriched with synthetic Bauer alkylamide 11 and Bauer ketone 23, and AK = combination of synthetic Bauer alkylamide 11 and Bauer ketone 23. (B) Standardized log signal for the two clusters identified in the analysis represents changes in gene expression level with different treatment groups. The number of probe sets is given in parentheses on the right above the cluster graph.

increase of COX-2, TNF- α , and iNOS genes after treatment with the medium + DMSO + LPS control compared to the medium + DMSO control. GADPH was selected as the housekeeping gene for the experiments, indicating no change in mRNA levels with any of the treatments during the time course from 0.5 to 24 h (data not shown). COX-1 mRNA was shown to increase at the 24 h time point after treatment with *E. angustifolia* fraction 3, enriched fraction 3, Bauer alkylamide 11, and Bauer ketone 23 compared to the medium + DMSO + LPS control, although the combination of Bauer alkylamide 11 and Bauer ketone 23 did not significantly change the mRNA levels.

Protein levels of COX-1, COX-2, and α -tubulin were assessed after the treatment with the *E. angustifolia* fraction 3, enriched fraction 3, and the combination of synthetic Bauer alkylamide 11 and Bauer ketone 23 (**Figure 6**), Bauer alkylamide 11 (**Figure 7**), and Bauer ketone 23 (**Figure 8**). From these analyses it was shown

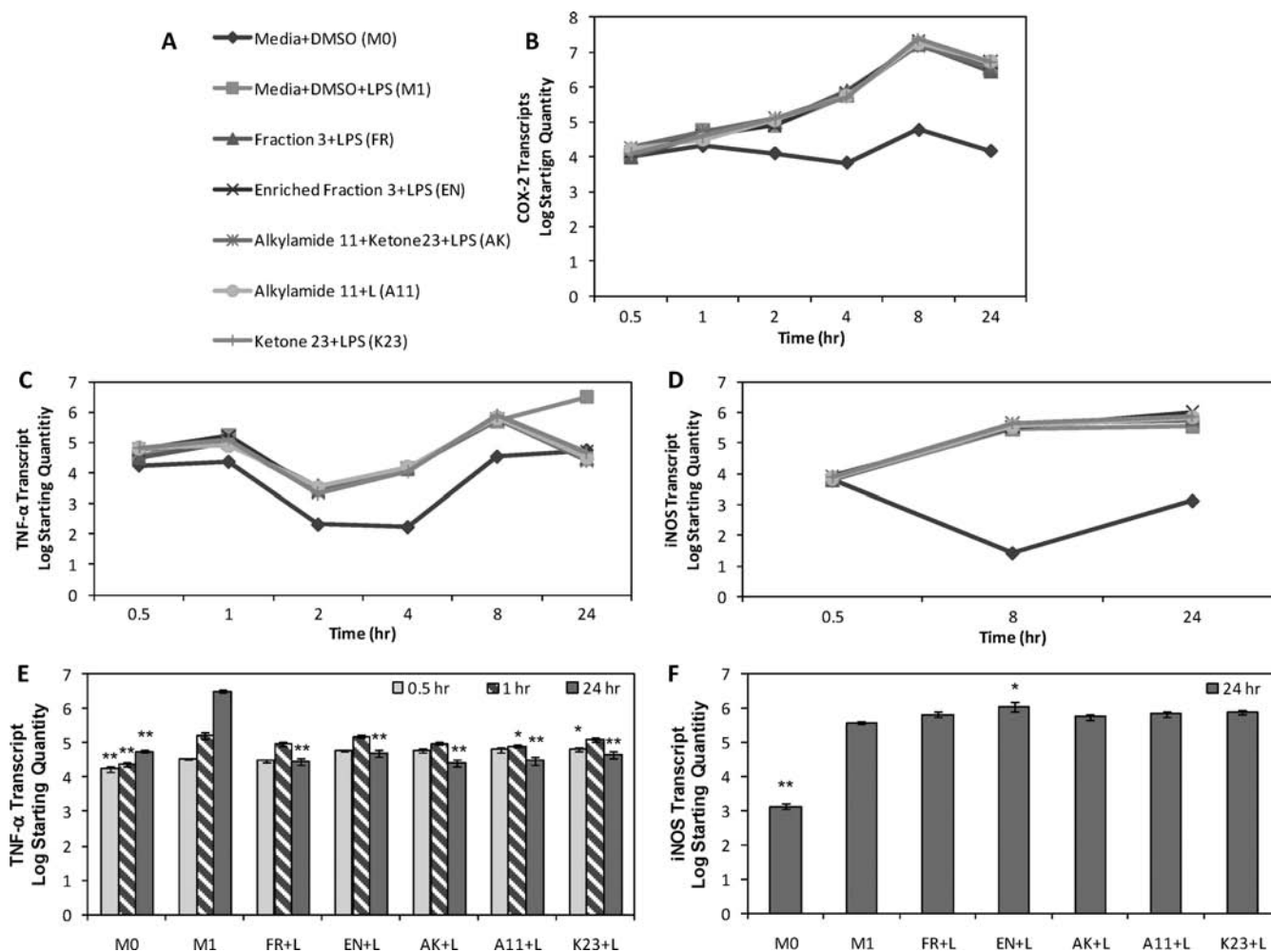


Figure 4. (A) Legend for qRT-PCR treatments. (B) Analysis of qRT-PCR time course for COX-2 gene expression. $N = 3$ for each treatment. Standard errors ranged from 0.02 to 0.16 TNF- α transcript log starting quantity for all treatments and time points. (C) Analysis of qRT-PCR time course for TNF- α gene expression. $N = 3$ for each treatment. Standard errors ranged from 0.03 to 0.16 TNF- α transcript log starting quantity for all treatments and time points. * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. (D) Analysis of qRT-PCR time course for iNOS gene expression. $N = 3$ for each treatment. Standard errors ranged from 0.03 to 0.24 for all treatments and time points. * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. (E) qRT-PCR analysis at time points with significant treatment effects for TNF- α gene when compared to the medium + DMSO + LPS control. * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. Bars represent the mean \pm standard error. (F) qRT-PCR analysis at time point with significant treatment effect for iNOS gene when compared to the medium + DMSO + LPS control. * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. Bars represent the mean \pm standard error.

that there was a significant increase in COX-2 protein with fraction 3 at 1 and 5 $\mu\text{g}/\text{mL}$, as well as with Bauer ketone 23 at 5 μM . Interestingly, there was a significant decrease in COX-1 protein with the treatment of fraction 3 at 5 $\mu\text{g}/\text{mL}$, yet α -tubulin protein level remained unchanged. Protein levels of LPS-induced iNOS were also assessed after treatment with enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually, showing no significant differences between treatments (ranging from 97.7 ± 23.5 to $101.1 \pm 21.7\%$ of control) and medium + DMSO + LPS control after a 24 h treatment.

COX-2 activity (Figure 9) was analyzed after treatment of the RAW264.7 macrophage cells for 8 h with *E. angustifolia* fraction 3 + LPS, enriched fraction 3 + LPS, the combination of Bauer alkylamide 11 and Bauer ketone 23 + LPS, and the synthetic constituents individually + LPS.

To further examine the effect of the *E. angustifolia* fraction 3, enriched fraction, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these compounds individually on the LPS-induced inflammatory response in the RAW264.7 mouse macro-

phage cell line at the 24 h treatment time, cell culture supernatants were analyzed on a multiplex assay system. Of the 32 cytokines/chemokines, 15 (including the TNF- α protein data presented above) showed significant treatment differences compared to the medium + DMSO + LPS control (Table 2). All cytokines/chemokines displayed in Table 2 except MIP-1b showed decreases in protein concentration with treatments including the synthetic Bauer constituents either alone or in combination + LPS compared to the medium + DMSO + LPS control. The enriched fraction + LPS significantly decreased IL-1b, IL-4, IL-12(p40), KC, LIF, LIX, and MIG protein levels compared to control (Table 2). *E. angustifolia* fraction 3 + LPS showed significant inhibition of LIF protein levels compared to control (Table 2). Many cytokines/chemokines showed a trend ($0.05 < p < 0.10$) toward significant decreases including the enriched fraction + LPS impact on Eotaxin, IL-7 MIP-1b, and RANTES.

DISCUSSION

A major finding in this study is that by enriching an *E. angustifolia* fraction (1 $\mu\text{g}/\text{mL}$) with Bauer alkylamide 11 (3.5 μM) and Bauer

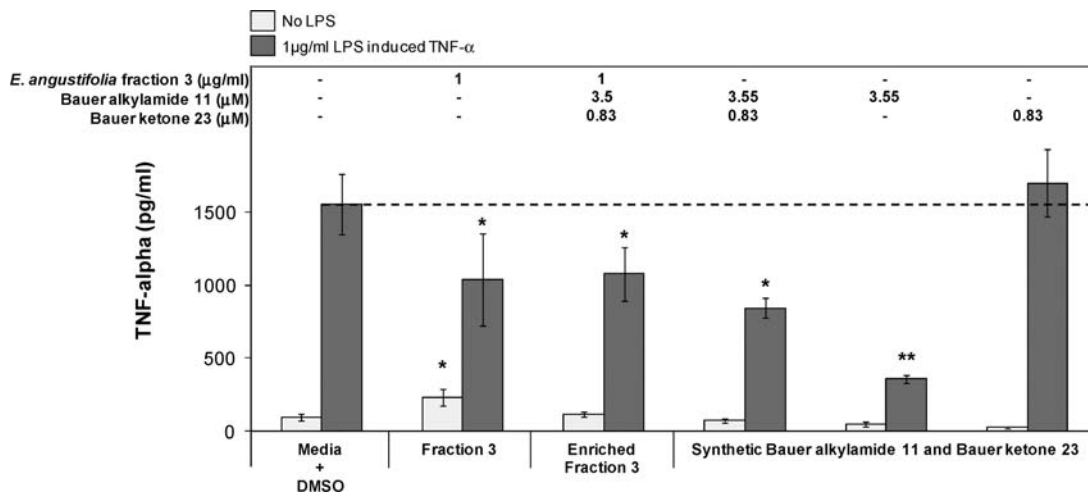


Figure 5. LPS-induced TNF- α production in RAW264.7 cells treated with *E. angustifolia* fraction, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and Bauer ketone 23. The light gray bars represent TNF- α levels after treatment with *Echinacea* fraction, enriched fraction or compounds. The dark gray bars represent TNF- α levels after induction with 1 μ g/mL LPS and treatment ($N=3$). All treatments + LPS were compared to medium + DMSO + LPS control. The treatments without LPS were compared to the medium + DMSO control. ($p < 0.0001$). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. Each bar represents mean \pm standard error.

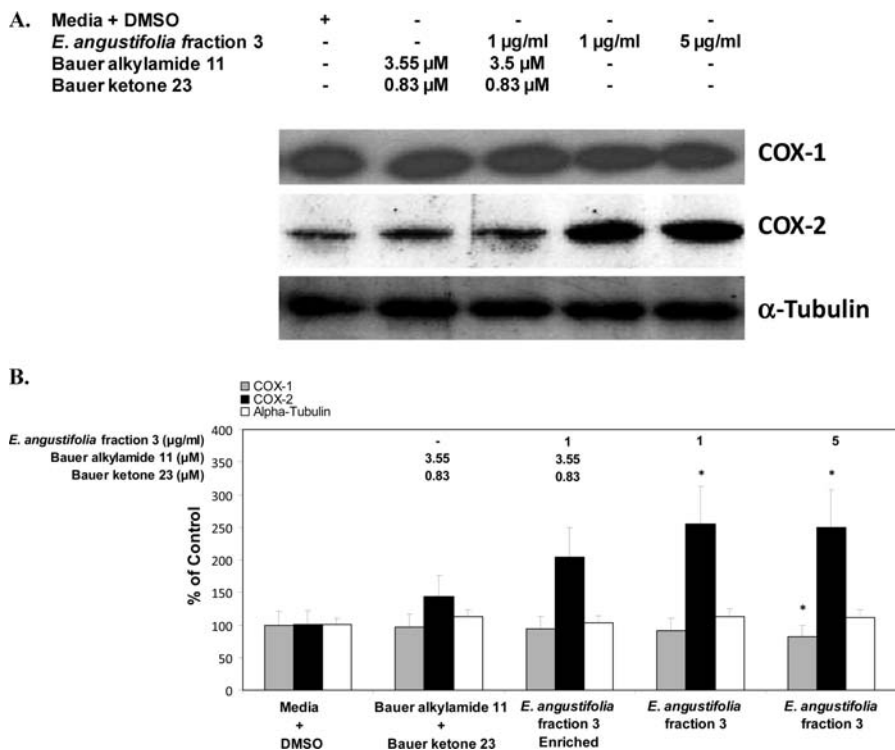


Figure 6. (A) Analysis of LPS-induced COX-1, COX-2, and α -tubulin protein levels in RAW264.7 cells with representative Western blots for *E. angustifolia* fraction 3, enriched fraction 3, and the combination of Bauer alkylamide 11 and Bauer ketone 23. $N=3$ for each blot. (B) Semiquantitative representation of the blots from A. Bars represent mean percent of medium + DMSO + LPS control \pm standard error. LPS-induced COX-2 protein from $24.3 \pm 8.2\%$ average for the medium + DMSO control to $100 \pm 20.6\%$ average for the medium + DMSO + LPS control. There was no significant LPS effect for the medium + DMSO control with COX-1 ($96.9 \pm 1.8\%$ of control average) or α -tubulin ($104.4 \pm 3.4\%$ of control average) on protein level compared to the medium + DMSO + LPS control. Quercetin was used as a positive control at 100 μ M for the reduction of LPS induced COX-2 protein $41.3 \pm 16.9\%$ of control. Quercetin did not significantly affect LPS-induced COX-1 ($85.3 \pm 3.2\%$ of control average) or α -tubulin ($109.6 \pm 16.7\%$ of control average). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively, when compared to medium + DMSO + LPS control.

ketone 23 (0.83 μ M) a more potent inhibition of the LPS-induced inflammatory mediator, PGE₂, was identified compared to that seen with the fraction alone after an 8 h treatment on the RAW264.7 macrophage cells. From these results, it appears that Bauer alkylamide 11 and Bauer ketone 23 accounted for a majority of the PGE₂

inhibitory capabilities of the fraction, but they did not show evidence of additivity. This finding indicates that it is possible to manipulate an *Echinacea* product to target a specific bioactivity after the discovery of constituents of importance as well as concentrations required to elicit the desired effect. Having the ability to target

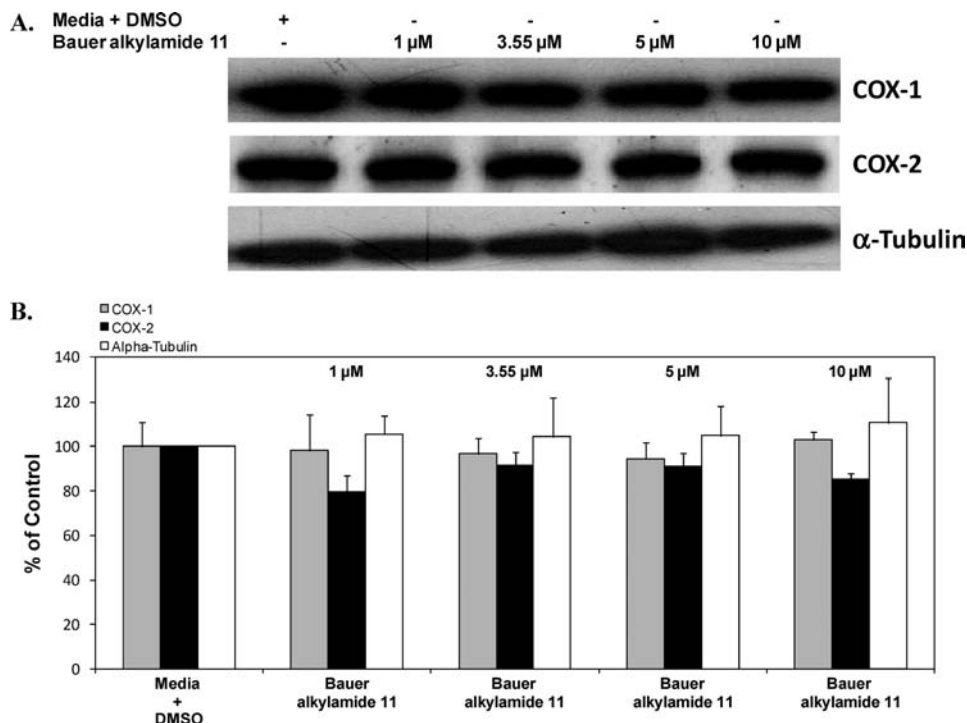


Figure 7. (A) Analysis of LPS-induced COX-1, COX-2, and α -tubulin protein levels in RAW264.7 cells with representative Western blots for Bauer alkylamide 11. $N = 3$ for each blot. (B) Semiquantitative representation of the blots from A. Bars represent mean percent of medium + DMSO + LPS control \pm standard error. LPS-induced COX-2 protein from $19.1 \pm 6.2\%$ average for the medium + DMSO control to $100 \pm 10.9\%$ average for the medium + DMSO + LPS control. There was no significant LPS effect for the medium + DMSO control with COX-1 ($83.4 \pm 3.0\%$ of control average) or α -tubulin ($94.7 \pm 2.1\%$ of control average) on protein level compared to the medium + DMSO + LPS control. Quercetin was used as a positive control at $100 \mu\text{M}$ for the reduction of LPS-induced COX-2 protein to $71.4 \pm 3.1\%$ of control. Quercetin did not significantly affect LPS-induced COX-1 ($96.1 \pm 6.5\%$ of control average) or α -tubulin (98.0 ± 4.3 of control average). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively, when compared to medium + DMSO + LPS control.

specific bioactivities with *Echinacea* products would be very important in the development of botanical products that are used for specific medicinal purposes. Major studies of the clinical efficacy of *Echinacea* to treat symptoms of the common cold or other upper respiratory infections have been ambiguous due to several confounding factors, including the use of different or undefined preparations of this botanical (25, 26). To characterize which constituents are necessary for particular medicinal outcomes and at what concentrations, studies are needed to provide concrete evidence of in vivo effectiveness in both animals and humans, as well as bioavailability of these constituents both individually and as a complex mixture.

Our studies set out to understand the mechanism of action leading to the inhibition of PGE₂ production in the RAW264.7 cells by *Echinacea* after an 8 h treatment period (12, 13). Using microarray analysis and qRT-PCR, it became apparent that our *Echinacea* treatments were not acting on the gene expression level for COX-2 or other potentially immune-related transcripts. However, Western blot analysis led to the intriguing finding that *E. angustifolia* fraction 3 and Bauer ketone 23 could increase COX-2 protein levels after an 8 h treatment, yet also have the ability to inhibit PGE₂ production at that same time point. These studies directed the way to another key observation, which is supported by previous literature (17), that the identified inhibition of LPS-induced PGE₂ production with *E. angustifolia* fraction 3, enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually on the RAW264.7 cells occurs in part through the inhibition of COX-2 activity. The discovery of COX-2-specific inhibitors has been the objective in the development of several drugs for relief of inflammatory symptoms. Aspirin was one of the first COX inhibitors

shown to act through acetylation of an active-site serine residue (27). Other COX inhibitors called nonsteroidal anti-inflammatory drugs (NSAIDs) act as competitive inhibitors of COX by reversibly engaging the binding site for arachidonic acid. Understanding the kinetics behind the inhibition of COX-2 activity with these *Echinacea* treatments is critical to understanding the plant's possible usefulness as an anti-inflammatory agent. The results obtained herein allow us to hypothesize a mechanism for the inhibition of PGE₂ production based on increased COX-2 protein levels and decreased COX-2 activity. One model that could explain these observations would be that these *Echinacea* products are directly inhibiting COX-2 activity by competing with arachidonic acid for the active site of this enzyme while at the same time blocking ubiquitination that would normally target the enzyme for degradation. This model is consistent with the observed decrease in COX-2 activity, decrease in PGE₂ production, and increase in COX-2 protein, with no change in gene expression.

TNF- α is a cytokine regulated through the cannabinoid receptors and, therefore, modulated through the binding of *Echinacea* alkylamides to the CB2 receptor. Gertsch et al. performed a RT-PCR time course experiment to understand how *Echinacea* modulated TNF- α gene expression in monocytes/macrophages (4). The results from this study showed that LPS-induced TNF- α mRNA decreased around 10 h after treatment of the alkylamide-rich preparation, with a steady decline until approximately 25 h. Other findings from this study indicated that it was the alkylamide constituents that acted on the CB2 receptors to modulate the TNF- α gene expression at a concentration as low as $0.5 \mu\text{M}$ (4). Recently, Chicca et al. provided further evidence that *N*-alkylamides were major contributors to the synergistic inhibitory effect on TNF- α identified upon combination of radix and herba tinctures acting via

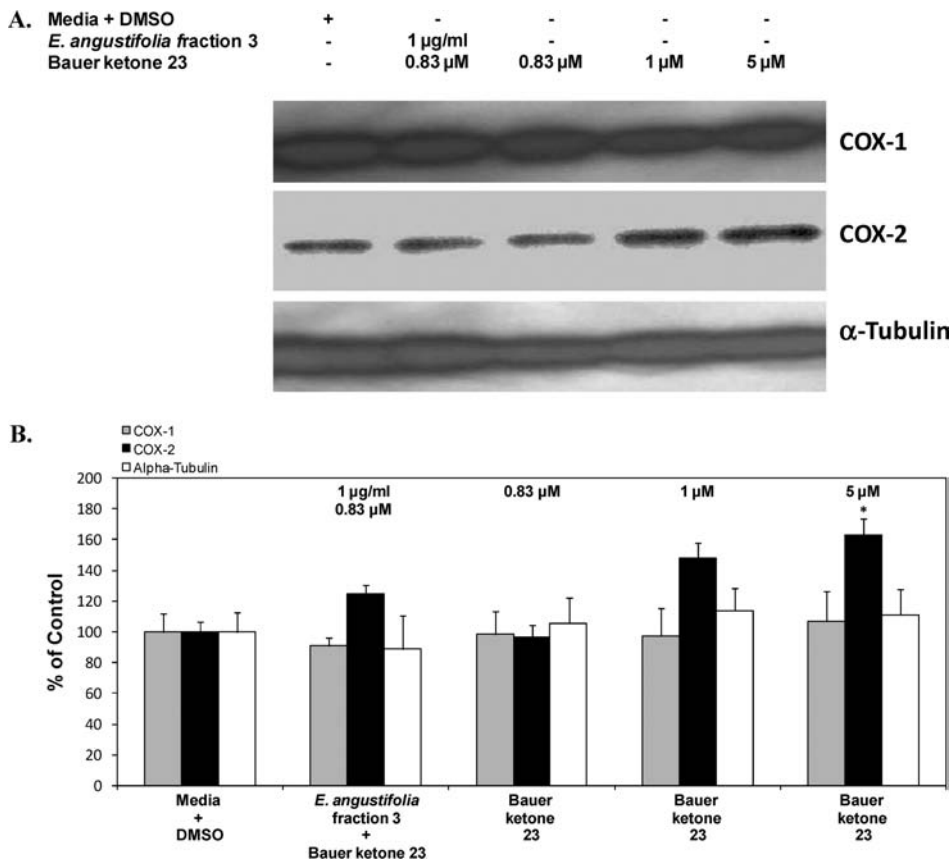


Figure 8. (A) Analysis of LPS-induced COX-1, COX-2, and α -tubulin protein levels in RAW264.7 cells with representative Western blots for Bauer ketone 23 individually and in combination with *E. angustifolia* fraction 3. $N = 3$ for each blot. (B) Semi-quantitative representation of the blots from A. Bars represent mean percent of medium + DMSO + LPS control \pm standard error. LPS induced COX-2 protein from $4.8 \pm 2.8\%$ average for the medium + DMSO control to $100 \pm 12.0\%$ average for the medium + DMSO + LPS control. There was no significant LPS effect for the medium + DMSO control with COX-1 ($95.4 \pm 3.3\%$ of control average) or α -tubulin ($103.9 \pm 7.3\%$ of control average) on protein level compared to the medium + DMSO + LPS control. Quercetin was used as a positive control at $100 \mu\text{M}$ for the reduction of LPS-induced COX-2 protein to $53.0 \pm 15.7\%$ of control. Quercetin did not significantly affect LPS-induced COX-1 ($95.5 \pm 2.2\%$ of control average) or α -tubulin (91.8 ± 9.7 of control average). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively, when compared to medium + DMSO + LPS control.

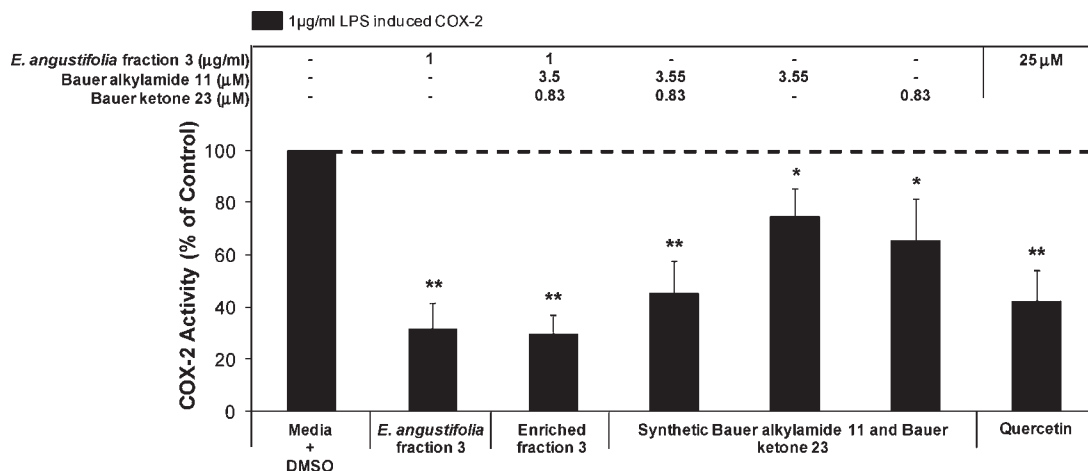


Figure 9. LPS-induced COX-2 activity in RAW264.7 cells treated with *E. angustifolia* fraction, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and Bauer ketone 23. The black bars represent COX-2 activity levels after induction of cells with $1 \mu\text{g/mL}$ LPS and treatment ($N = 3$). All treatments with added LPS were compared to a medium + DMSO + LPS control that was set at 100% COX-2 activity (5.8 nmol/min/mL). The treatments without LPS were compared to the medium + DMSO control set at 100% COX-2 activity (2.4 nmol/min/mL), identifying no significant differences. Quercetin was used as a positive control for both studies and showed significant inhibition of COX-2 activity ($p < 0.0001$). * and ** are representative of $p < 0.05$ and $p < 0.01$, respectively. Each bar represents percent of control \pm standard error.

CB2 receptors (28). Our qRT-PCR time course was carried out to 24 h, showing a significant decrease in LPS-induced TNF- α gene

expression with the alkylamide-rich *E. angustifolia* fraction 3, enriched fraction 3, combination of alkylamide and ketone, and

Table 2. Milliplex Data Examining Accumulation of 15 Cytokines/Chemokines in RAW264.7 Cells^a

cytokine/chemokine	protein concentrations after 24 h treatment to RAW264.7 cells					
	medium + DMSO + LPS	<i>E. angustifolia</i> fraction 3 + LPS	enriched fraction 3 + LPS	Bauer alkylamide 11 and Bauer ketone 23 + LPS	Bauer alkylamide 11 + LPS	Bauer ketone 23 + LPS
Eotaxin (pg/mL)	56.6 ± 4.5	48.3 ± 0.9 0.209	43.8 ± 5.0 0.067	45.8 ± 2.2 0.113	42.0 ± 4.8 0.041	39.0 ± 3.4 0.018
IL-1b (pg/mL)	220.1 ± 46.4	142.6 ± 5.8 0.052	120.2 ± 13.9 0.017	152.0 ± 5.2 0.081	114.6 ± 20.1 0.013	81.0 ± 12.7 0.003
IL-4 (pg/mL)	7.0 ± 0.6	6.0 ± 0.2 0.225	4.8 ± 0.4 0.017	5.8 ± 0.4 0.1614	5.0 ± 0.6 0.024	4.5 ± 0.3 0.008
IL-6 (ng/mL)	24.6 ± 0.2	24.2 ± 0.2 0.821	23.8 ± 0.2 0.621	24.4 ± 0.1 0.886	23.3 ± 0.6 0.467	19.8 ± 2.1 0.017
IL-7 (pg/mL)	8.7 ± 0.6	7.1 ± 0.6 0.270	6.1 ± 0.5 0.088	6.4 ± 0.8 0.127	5.3 ± 1.6 0.032	4.1 ± 0.1 0.007
IL-12 (p40) (pg/mL)	22.8 ± 5.0	7.7 ± 5.0 0.051	7.4 ± 1.4 0.050	7.5 ± 3.2 0.048	22.0 ± 0.1 0.764	2.7 ± 0.6 0.016
IL-15 (pg/mL)	86.6 ± 14.6	58.7 ± 2.4 0.119	57.6 ± 6.7 0.107	63.0 ± 5.5 0.180	48.2 ± 15.1 0.041	43.5 ± 5.7 0.025
KC (pg/mL)	352.6 ± 28.1	269.7 ± 7.8 0.110	216.2 ± 24.4 0.016	283.2 ± 38.7 0.173	245.3 ± 27.3 0.047	228.8 ± 34.2 0.026
LIF (ng/mL)	1.9 ± 0.3	1.2 ± 0.1 0.019	0.9 ± 0.2 0.005	1.2 ± 0.1 0.021	0.8 ± 0.2 0.002	0.6 ± 0.2 0.001
LIX (ng/mL)	5.2 ± 0.6	4.5 ± 0.3 0.356	3.6 ± 0.4 0.038	4.3 ± 0.4 0.194	3.3 ± 0.7 0.018	3.9 ± 0.5 0.0897
MCP-1 (ng/mL)	14.1 ± 0.3	12.8 ± 363.9 0.391	12.9 ± 0.7 0.422	1.3 ± 628.4 0.659	9.9 ± 1.6 0.015	13.2 ± 0.8 0.558
MIG (pg/mL)	26.0 ± 5.3	17.8 ± 1.5 0.114	15.0 ± 1.2 0.044	21.3 ± 1.0 0.347	13.0 ± 3.7 0.021	15.4 ± 3.1 0.050
MIP-1a (ng/mL)	10.5 ± 0.4	7.5 ± 1.2 0.144	9.5 ± 0.6 0.279	7.7 ± 2.0 0.5871	6.8 ± 1.8 0.021	10.2 ± 0.03 0.558
MIP-1b (ng/mL)	38.9 ± 11.1	17.8 ± 4.0 0.129	11.7 ± 0.9 0.075	45.1 ± 20.0 0.938	30.6 ± 20.3 0.1051	26.0 ± 5.9 0.2614
RANTES (ng/mL)	3.2 ± 0.6	2.0 ± 0.5 0.112	1.7 ± 0.3 0.053	1.9 ± 0.3 0.095	0.9 ± 0.1 0.006	1.7 ± 0.1 0.046

^a Milliplex analysis examined 32 cytokines/chemokines. Only cytokines/chemokines that are known to be strongly relevant to the macrophage response to inflammation are included in the table. Each treatment was compared to the medium + DMSO + LPS control. Only LPS-treated samples are displayed. Top numbers in table represent mean of three replicates ± standard error and bottom numbers indicate *p* values compared to the value for the medium + DMSO + LPS control. Boldface *p* values represent statistical significance (*p* < 0.05).

the individual constituents, corresponding with these earlier findings (4, 28), although the concentration of alkylamide in our studies was slightly higher at 3.55 μM than that reported the study by Gertsch et al. (4). Our studies indicate that these treatments, excluding Bauer ketone 23, also significantly inhibited TNF-α protein production. Due to the similarity in our results it could be hypothesized that our *Echinacea* treatments are also acting through binding the cannabinoid receptors, with the exception of synthetic Bauer ketone 23 (0.83 μM), which also showed a significant decrease in TNF-α gene expression at the 24 h time point. An interesting finding from our studies is that Bauer ketone 23 (0.83 μM) alone did not significantly modulate TNF-α protein levels. Egger et al. determined that ketones, analyzed at 3% (v/v) in DMSO, do not appear to mediate their immunomodulatory effects through the cannabinoid receptors (3), and therefore it is likely that

Bauer ketone 23 acts through a different mechanism to inhibit the noted TNF-α gene expression identified in our qRT-PCR studies after a 24 h treatment, although these findings do not rule out the possibility that Bauer ketone 23 may interfere indirectly with the endocannabinoid system in a manner that could lead to the inhibition of TNF-α expression.

The results obtained from the multiplex assay would indicate that treatments including the enriched fraction, the combination of Bauer alkylamide 11 and Bauer ketone 23, or these constituents individually act in an immunosuppressive manner, with the ability to inhibit the accumulation of several cytokines/chemokines. The inhibition of chemokines may limit the recruitment of specific cell populations. For example, reduction of KC may reduce neutrophil migration, whereas a decrease in LIX, MCP-1, MIP-1α, and MIP-1β may limit recruitment of inflammatory monocytes/

macrophages. In general, a decrease in cytokines may decrease cellular activation. A decline in IL-4 may inhibit T-helper cell type 2 response, whereas a decline in IL-12 and MIG may limit T-helper cell type 1/IFN γ -induced cell-mediated immune responses. Taken together, the reduction in these multiple chemokines/cytokines may have beneficial effects in vivo at a resolution phase of infection when the inflammatory immune response should decline.

LPS-induced NO production was significantly inhibited by treatments of synthetic Bauer alkylamide 11 and Bauer ketone 23, Bauer alkylamide 11 individually, and Bauer ketone 23 individually after a 24 h incubation on the RAW264.7 mouse macrophage cells. From the gene expression data it was determined that the enriched fraction 3 showed a significant increase in iNOS mRNA compared to the medium + DMSO + LPS control, although NO production was not increased. Enriched fraction 3, Bauer alkylamide 11, and Bauer ketone 23 were shown to have no effect on LPS-induced iNOS protein levels after a 24 h incubation. Previous studies on *Echinacea* extract treated RAW264.7 cells incubated for 23 h determined that the extracts were capable of NO inhibition, not through direct scavenging of the free radical but through the inhibition of iNOS (15). Chen et al. also demonstrated that alkylamides were capable of inhibiting LPS-induced NO production after a 24 h incubation in the RAW264.7 cell line, including isolated Bauer alkylamide 11, with an ID₅₀ of 23.9 μ M (14). Our prior studies had also indicated that Bauer ketones could significantly inhibit NO production at concentrations as low as 1 μ M (12). The findings from the present study indicate that compounds from *Echinacea* target NO production, and future studies will concentrate on the mechanism leading to the identified inhibition.

Although immunostimulatory effects have been identified in other studies using water extracts of *Echinacea* (2), it is important to point out that the results acquired from this study provide evidence that through the enrichment of an *Echinacea* fraction with the addition of key anti-inflammatory constituents, it is possible to enhance the anti-inflammatory potential of this botanical. It is also of interest to note that no additive or synergistic effects were identified with treatments that combined synthetic Bauer alkylamide 11 and Bauer ketone 23 in the PGE₂ screening, although the addition of these compounds to the *E. angustifolia* fraction proved to increase anti-inflammatory potential. This research also has led to a proposed mechanism in which *Echinacea* fractions and pure constituents may inhibit PGE₂ and increase COX-2 protein expression by competitively binding the COX-2 enzyme while blocking ubiquitination and, therefore, degradation of the protein. The model described was established on the basis of the results obtained from our COX-2 protein and activity studies indicating that enriched fraction and synthetic Bauer ketone 23 increased COX-2 protein level and the *E. angustifolia* fraction, enriched fraction, combination of constituents, and individual constituents were all capable of a significant inhibition of COX-2 enzyme activity, therefore accounting for the identified inhibition of PGE₂ production.

ABBREVIATIONS USED

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.

SAFETY

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

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