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Identification of key anti-inflammatory *Echinacea* constituents and their mechanism leading to the inhibition of prostaglandin E2

by

Carlie Ann LaLone

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Diane Birt, Major Professor
Marian Kohut
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Ames, Iowa

2009

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ABSTRACT

Preparations of *Echinacea* as a dietary supplement are most commonly used to treat or lessen the severity of symptoms associated with the common cold or other upper respiratory tract infections, although the use *Echinacea* as a medicinal herb dates back hundreds of years when Native American peoples used it to treat various ailments ranging from snake bite antidotes to venereal diseases to rheumatism. With the popularity of this botanical growing throughout the United States, and the world, studies have been ongoing to understand how *Echinacea* and its constituents modulate the immune system.

The purpose of this study was to identify *Echinacea* species, fractions, and constituents responsible for the anti-inflammatory properties associated with *Echinacea*, as well as establish a mechanistic basis for these properties. The model system chosen to carry out these studies was the RAW264.7 mouse macrophage cell line which is a strongly established model used to mimic the inflammatory response when induced with lipopolysaccharide (LPS). Inhibition of the production of the pro-inflammatory lipid mediator, prostaglandin E2 (PGE_2), along with parallel cytotoxicity studies were used to identify *Echinacea* species with the greatest anti-inflammatory potential. From the screening of Soxhlet ethanol root extractions (prepared in Dr. Patricia A. Murphy's laboratory) from six of the nine *Echinacea* species, it was determined that *Echinacea angustifolia*, *Echinacea pallida*, *Echinacea simulata*, and *Echinacea sanguinea* were the strongest inhibitors of LPS induced PGE_2 production, showing no cytotoxic effects. Common alkylamides of *Echinacea* were also chemically synthesized in Dr. George A. Kraus's laboratory and examined for their ability to inhibit PGE_2 production, identifying 13 alkylamides capable of significant inhibition of the lipid mediator at 50 μM, five alkylamides capable of significant inhibition at 25 μM, and only one alkylamide, Bauer alkylamide 14, capable of significant inhibition at 10 μM. Again, these constituents of *Echinacea* did not show cytotoxic effects at concentrations

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at or below 50 μM. Alkylamides present in the *Echinacea* species extracts were present at concentrations much lower than those screened in the PGE_2 assay, implying that although alkylamides are anti-inflammatory as synthetic constituents, other constituents present in the plant, either previously identified or not, or interactions among these constituents are important for the anti-inflammatory properties of *Echinacea* preparations.

In order to unravel the complex mixture of *Echinacea* constituents to identify key contributors to the anti-inflammatory activity, bioactivity guided semi-preparative reverse phased HPLC was used to fractionate four species of *Echinacea* in Dr. Patricia A. Murphy's laboratory. From the fractionation of an *Echinacea pallida* extract, it was determined that Bauer ketones 23 and 24 were important for the identified PGE_2 inhibitory capabilities of a ketone rich first round fraction, necessitating further study of this group of compounds for their anti-inflammatory potential. Following the inhibition of PGE_2 production through three rounds of fractionation with an *Echinacea angustifolia* extract led to the identification of Bauer alkylamide 11 and Bauer ketone 23 at concentrations present in their respective third round fractions capable of partially explaining the PGE_2 inhibition observed prior with their corresponding fraction. Synthetic Bauer alkylamide 11 was also capable of significant inhibition of nitric oxide production. The knowledge that Bauer alkylamide 11 and Bauer ketone 23 were key contributors to the anti-inflammatory properties of *Echinacea* at endogenous concentrations led to the hypothesis that through the enrichment of a first round alkylamide rich fraction of *E. angustifolia* with synthetic Bauer alkylamide 11 and Bauer ketone 23 an enhanced anti-inflammatory potential could be achievable. By enriching the fraction with synthetic Bauer alkylamide 11 and Bauer ketone 23 to concentrations determined to be important from the third round fractions, a greater inhibition of PGE_2 production was identified than that observed with the fraction alone.

In order to identify key gene targets for the *Echinacea angustifolia* fraction, enriched fraction, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these

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constituents individually, microarray and time course qRT-PCR studies were conducted. These studies indicated from the microarray study that the selected *Echinacea* treatments to LPS induced RAW264.7 cells did not alter gene expression after eight hours, with only eight differentially expressed (DE) genes being identified with false discovery rates (FDR) ranging from 50% to 75% in the microarray. Although, 3,257 DE genes were identified between the media + DMSO and the media + DMSO + LPS controls with a FDR of 0.0001%, establishing the expected LPS effect. The qRT-PCR data showed a decrease in TNF-α gene expression after treatment with all samples and an increase in iNOS expression after treatment with enriched fraction. Although PGE_2 production had been decreased by these treatments, COX-2 mRNA levels were not significantly different between treatments compared the media + DMSO + LPS at any time point analyzed (0.5 hr, 1 hr, 2hr, 4 hr, 8 hr, and 24 hr). COX-2 protein levels were increased after an 8 hour treatment of *E. angustifolia* fraction 3, and Bauer ketone 23. Furthermore the activity of COX-2 was inhibited by all treatments. Combined these studies suggest that *Echinacea* extracts, fractions and certain classes of constituents have anti-inflammatory potential through the direct modulation of a key enzyme in the eicosanoid pathway and that the noted inhibition of NO production and TNF-α gene expression may be due to the effect these treatments have on divergent signal transduction pathways.

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CHAPTER 1: GENERAL INTRODUCTION

Echinacea (purple cone flower) is a member of the Asteraceae family that includes other species such as *Ambrosia* (ragweed), *Artemisia* (mugwort, sagebrush, and wormwood), Parthenium (feverfew), sunflowers, safflower, dahlias, chrysanthemums, marigolds, and daisies (*1*). It is a perennial flower native to the central United States and commonly found as an ornamental flower in gardens of this region, although cultivation of this botanical has now spread worldwide (*2*). The nine species of *Echinacea* that have been characterized are *E. purpurea*, *E. angustifolia*, *E. pallida*, *E. sanguinea*, *E. simulata*, *E. tennesseensis*, *E. laevigata*, *E. atrorubens*, and *E. paradoxa*, with each consisting of its own unique phytochemical make-up (*3*). The diversity, or in some cases similarity, of the constituents present in these *Echinacea* species not only aid in the identification of each species, but also contributes to the diverse bioactivities associated with the botanical *Echinacea*. Three species, *E. purpurea*, *E. angustifolia*, and *E. pallida*, are commonly classified as the medicinal species due to their early use by American Indians hundreds of years prior and because of their current presence in commercial preparations worldwide (*4*, *5*).

Approximately 400 years ago American Indian tribes, such as the Cheyenne, Choctaw, Dakota, Delaware, Fox Kiowa, Montana, Omaha Pawnee, Ponca, Sioux, and Winnebago, discovered the medicinal properties of the *Echinacea* plant (*5*). Information regarding the uses of *Echinacea* and other botanicals by Native Americans has been made available through an Internet database (http://herb.umd.umich.edu/) that has taken years to compile (*6*). *Echinacea* roots and aerial parts were used to treat a wide range of ailments, such as toothaches, sore throat, pain reliever, headache, antidote for poisons, and to relieve swelling, just to name a few (*5*).

Today *Echinacea* preparations are most commonly used for the treatment or prevention of common cold symptoms or other respiratory tract infections (*3*). Extracts of

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the whole *Echinacea* plant, root, and/or aerial parts are available in a variety of different forms, including but not limited to pills, tablets, liquids, teas, tinctures, and toothpastes (*7*). *Echinacea* products are quite popular in the United States with this botanical remaining in the top 10 selling herbal dietary supplements (*8*).

Echinacea extracts have been studied for several bioactivities and have yielded encouraging results as anti-inflammatory, anti-viral, immunomodulatory, and anti-oxidant agents (*9-12*). Five classes of constituents found in the *Echinacea* species are presumed to be responsible for much of the bioactivity of this botanical, those being, alkylamides, caffeic acid derivatives, polysaccharides, glycoproteins, and more recently ketones (*9*). Alkylamides have been hotly followed due to their reported immunomodulatory and anti-inflammatory properties *(13)* and caffeic acid derivatives are better known for their anti-oxidant capabilities *(14)*. Polysaccharides were shown to have macrophage activating properties *(15)* and glycoproteins have been studied for their immunostimulatory effects, such as the activation of the complement system *(16)*. Finally, ketones have shown promising results as cytotoxic agents toward certain tumor cells *(17)*. The reality is that researchers continue to discover diverse bioactivities that are in some way modulated by the constituents present in *Echinacea* products and further research is certainly warranted in order to clearly understand the complex mixture of these compounds making up this botanical and how they may act alone or synergistically.

An interesting topic that somewhat plagues researchers studying botanicals such as *Echinacea* is that of synergy and how to uncover the details of which constituents act in concert to produce a certain bioactivity *(18)*. In several studies it has been shown that certain constituents are capable of eliciting a desired response, but unfortunately when comparing the concentration of the compound with that present in an extract or other natural preparation, more often than not the endogenous concentration of that compound is much lower than that required for the bioactivity *(19)*. This adds to the complexity of the studies

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by leaving open the possibility for synergistic effects between the different constituents, whereby perhaps adding together the correct combination of compounds at the concentration attainable by the plant in nature, the bioactivity could be explained. Researchers have been using bioactivity guided fractionations to probe this idea of synergy between compounds. Part of this dissertation also used the method of bioactivity guided fractionation to delve into the complex mixture of constituents that makes up the *Echinacea* root extracts and will be further described in chapter 3.

Bioavailability has been of major concern in studies on *Echinacea* as well as other botanicals, with the question of "how much of the active compounds actually enter the body or cell and are able to elicit their effect?" Studies performed within the past decade utilizing the Caco-2 monolayer model and clinical trials, which will be described in detail in chapter 2, have started to build convincing evidence that alkylamides and polyacytelenes are bioavailable, whereas caffeic acid derivatives are to a lesser extent *(19-21)*.

Due to the fact that *Echinacea* species have been identified as immune modulators and people are utilizing this herb most prevalently to prevent or treat common cold symptoms *(22)*, an appropriate effect to examine would be that of inflammation, which was the main focus of this dissertation. Chapter 2 in this dissertation provides the necessary background to understanding the mounting of an inflammatory response, important cell types and cytokines involved in the inflammatory response, as well as what inflammatory mediators and signaling pathways may be of importance when studying the effects *Echinacea* products have on inflammation.

The RAW264.7 mouse macrophage cell line is a strongly established cell model for the study of inflammatory endpoints and was therefore chosen as a relevant system to explore the anti-inflammatory effects of *Echinacea* preparations *(11, 12)*. RAW264.7 cells are macrophage-like cells that were Abelson murine leukemia virus transformed from BALB/c mice. The macrophage cell was a logical choice for studying the innate immune response

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and has been proven to induce prostaglandin E2 production via lipopolysaccharide ligand binding.

The studies described in this dissertation were based on the hypothesis that *Echinacea* extracts, fractions, and pure constituents, such as alkylamides and ketones, are capable of inhibiting prostaglandin E2 production through the inhibition of the NF-kB signal transduction pathway, which therefore inhibits the expression of COX-2 in a RAW264.7 mouse macrophage model.

Thesis Organization

The arrangement of this thesis begins with a general introduction on *Echinacea's* history and common uses and the general ideas driving the research for this dissertation, followed by chapter 2, a literature review that delves into an explanation of the inflammatory response and provides a thorough evaluation of the research conducted thus far to understand the bioactivities associated with *Echinacea* extracts, fractions, and constituents. Chapter 3 consists of a manuscript published in the *Journal of Agricultural and Food Chemistry* entitled "*Echinacea* species and alkylamides inhibit prostaglandin E2 production in RAW264.7 mouse macrophage cells." Co-authors on this manuscript include: Kimberly D. P. Hammer, determined which solvents were appropriate for the extracts of the *Echinacea* species and aided in conducting PGE_2 experiments; Lankun Wu, conducted all HPLC experiments to identify constituents; Jaehoon Bae, synthesized all synthetic alkylamides; Norma Leyva, provided statistical consultation; Yi Liu and Avery K. S. Solco, prepared the extracts of the *Echinacea* species; George A. Kraus, mentored Jahoon Bae and guided the chemical synthesis of the alkylamides; Patricia A. Murphy mentored and consulted Yi Liu and Avery K. S. Solco on the extraction process for the *Echinacea* species extracts; Eve S. Wurtele, mentored Lankun Wu on the HPLC analyses; Ok-Kyung Kim, measured cytotoxicity on three species extracts; Kwon II Seo, conducted the cytotoxicity studies with the NIH/3T3 cells, SW480 cells, and HaCaT cells; Mark P. Widrlechner, provided all

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Echinacea plant material; and Diane F. Birt, mentored Carlie A. LaLone and provided guidance and edits to all research conducted and manuscript drafts. Chapter 4 contains a manuscript published in the *Journal of Agricultural and Food Chemistry* entitled "Endogenous Levels of *Echinacea* Alkylamides and Ketones are Important Contributors to the Inhibition of Prostaglandin E2 and Nitric Oxide Production in Cultured Macrophages." Co-authors on this manuscript included: Ludmila Rizshsky, conducted all GC-MS analyses; Kimberly D. P. Hammer, provided guidance on the progress of PGE_2 studies; Lankun Wu, conducted HPLC analysis on second round *E. angustifolia* fractions 3D and 3E; Avery K. S. Solco, conducted fractionations of *E. purpurea*, *E. angustifolia*, and *E. pallida*; Man-Yu Yum, provided statistical consultation; Basil J. Nikolau, provided GC-MS consultation; Eve S. Wurtele, mentored Lankun Wu on HPLC studies, Patricia A. Murphy, mentored Avery K. S. Solco on fractionation procedures; Meehye Kim, fractionated E. tennesseensis extract; and Diane F. Birt, mentored Carlie A. LaLone and provided guidance and edits to all research conducted and manuscript drafts. Chapter 5 is made up of a manuscript, to be submitted to the *Journal of Agricultural and Food Chemistry* entitled "Enrichment of *Echinacea angustifolia* with Bauer alkylamide 11 and Bauer ketone 23 increased anti-inflammatory potential through interfering with COX-2 activity." Co-authors on this manuscript included: Nan Huang, aided in running qRT-PCR experiments; Ludmila Rizshsky, conducted all GC-MS experiments; Man-Yu Yum, provided statistical consultation, Navarozedeep Singh, ran milliplex assay; Cathy Hauck, fractionated E. angustifolia extract; Basil J. Nikolau provided GC-MS consultation, Eve S. Wurtele, provided micro-array consultation; Marian L. Kohut, mentored Navarozedeep Singh during milliplex assay and provided immunological expertise; Patricia A. Murphy, provided fractionation expertise; and Diane F. Birt, mentored Carlie A. LaLone and provided guidance and edits to all research conducted and manuscript drafts. Finally, chapter 6 contains a general conclusion.

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CHAPTER 2: LITERATURE REVIEW

Inflammation and Inflammatory Diseases

Heat, swelling, redness, pain, and loss of function are known to be the cardinal signs of inflammation, as described by the Roman writer Celsus in the first century AD (*1*), but below the surface of these symptoms lies a complex network of inflammatory mediators and immunological signaling molecules. The inflammatory response plays three primary roles in the body to defend it from foreign antigens. Inflammation allows for the maturation of monocytes circulating in the blood stream into effector macrophage cells that enter the site of inflammation providing a first defense against invading organisms (*2*). Secondly, the inflammatory response inhibits the spread of the foreign antigen by causing microvascular coagulation (*2*). The third major role of inflammation is to promote the healing of damaged tissue (*2*). Key inflammatory mediators, such as lipid derivatives, oxygen and nitrogen radicals, and chemokines and cytokines are present at each step to guide the inflammatory process through its various stages and this will be the primary focus of the literature reviewed dealing with inflammation.

Role of Macrophage in Inflammation

The macrophage cell plays a dual role in the immune system acting in both innate and adaptive immune responses, as a phagocytic cell and antigen-presenting cell, respectively. This cell type has an important role in the inflammatory response leading the way to the cardinal symptoms of inflammation described previously, as well as to the resolution of the inflammatory response. The macrophage is instrumental in the development of each of these processes.

Macrophage cells are found in several locations throughout the body that act as barriers to the outside environment. They can be found in epidermal tissues, in the lungs, in connective tissue, and in liver tissue (kupffer cells) (*2*). The macrophages are in these

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strategic locations so that they can act as "watchdogs" guarding the body from any foreign invaders. When an antigen enters the body, perhaps through a wound in the skin, macrophage cells are present to engulf the antigen (phagocytosis) and release pro-inflammatory cytokines (Interleukin-1, Interleukin-6, Tumor Necrosis Factor-α, and Interleukin-12) and chemokines (Monocyte Chemoattractant Protein, Interleukin-8) that signal other innate immune cells to aid in the body's defense at the site of antigen infiltration and therefore inflammation (*3*). Different cell surface molecules such as selectins (E and D) and intracellular adhesion molecules (ICAM 1 and 2) are then expressed on the surface of epithelial cells located in the veins (*4*). Neutrophils, as well as monocytes have cell surface molecules, like leukocyte functional antigen-1 (LFA-1), that cause them to start rolling along the blood vessel and eventually extravasate out of the blood vessels following a chemokine gradient (IL-8), homing to the site of inflammation (*5*). Receptors play a key role in leading the various immune cells to key locations to set up a controlled inflammatory response.

Monocytes, which are immature macrophages that arise through the differentiation of progenitor cells in the bone marrow prior to their release into the blood stream, circulate in the blood until they are signaled into tissue to aid in innate immunity (*6*). This infiltration of cells causes the redness, swelling, heat, and pain associated with inflammation. In addition, the cytokines released from the macrophages cause vasodilation making the entrance of the other phagocytic cells more rapid, setting up an army of cells capable of destroying the invading antigen. Inflammation also causes clotting in the microvessels around the site of inflammation to contain the pathogen (*2*). Macrophages have receptors, such as mannose binding receptors, toll like receptors, and scavenger receptors that are capable of recognizing pathogen associated membrane patterns (*6*). These receptors allow macrophages to identify invading bodies and phagocytose them as well as initiate inflammatory pathways such as nuclear factor κappa B (NF-κB) and mitogen activated protein kinase (MAPK) that lead to the expression of pro-inflammatory genes and genes involved in adaptive immunity.

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Macrophages are able to release reactive oxygen species, such as nitric oxide within their phagocytic vesicles, which act to destroy the contents. Nitric oxide has been shown to be responsible for the activation of macrophage apoptosis, macrophage cytoplasmic motility, neutrophil adhesion, and regulation of cytokine synthesis (*7*). Macrophages also recognize antigen coated with complement and are able to take up and destroy these particles. What has been described thus far occurs locally at the site of inflammation, but the macrophage is also activating adaptive immune responses outside of the area of local inflammation.

Macrophage cells are one of the three main cells capable of presenting antigen, meaning that they are able to process foreign peptides that are engulfed through the process of phagocytosis and present those peptides on major histocompatability complexes (MHC) to T cells. The interaction between T cell receptors, MHC antigen, and B7 co-stimulatory molecules on macrophage cells (CD80 and CD86) allow for the activation of T cells, forming either CD4 or CD8 T cells (*2*). Macrophages play a role in the activation of more cells that will eventually aid in the site of inflammation, clearing up any foreign particles that could not be eliminated by innate immunity. The CD4 cells come in two varieties, TH1 cells that activate lysosomes within macrophages to kill intracellular organisms have been phagocytosed and TH2 cells that activate B cells to produce antigen that can bind to foreign molecules either neutralizing, preparing for opsonizing, or activating complement. These cells then hone to the site of inflammation and aid in clearing the foreign invader and therefore resolving the inflammatory response. Two separate studies have shown that depletion of macrophages, either using antimacrophage serum and hydrocortizone in a guinea-pig model or in the PU.1-null mouse model (PU.1 is a transcription factor that plays a key role in the development of myeloid lineages and therefore prevents macrophage development) led to the failure to clear dead and damaged cells and other debris at the wounded site, providing convincing evidence of the important role the macrophage cell plays in resolution of the inflammatory response (*8*).

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Eicosanoids Role in Inflammation

The eicosanoid cascade can be activated during an inflammatory response releasing arachidonic acid (AA), which is a dietary derived polyunsaturated C_{20} fatty acid (metabolized from linoleic acid and α-linolenic acid) that is normally stored in the cell phospholipid membrane (*9*). Arachidonic acid is liberated from the lipid membrane by phospholipase A2 enzymes, of which there are 3 main classes: secreted ($sPLA_2$), intracellular ($iPLA_2$), or cytosolic ($cPLA_2$), to assume three possible fates, either reincorporation into phospholipid, diffusion out of the cell, or metabolism (*10*). Experimental evidence has highlighted $cPLA_2$ as an integral component in the production of prostaglandins, known mediators of inflammation. $cPLA_2$ knockout mice show a decreased occurrence and less intense collagen-induced arthritis (*11*) and $cPLA_2$-deficient mice were shown to have decreased eicosanoid production (*12*). Due to the crucial role of phospholipase A2 enzymes in the production of prostaglandins, it has been a target for inhibition of these inflammatory mediators.

The metabolism of AA can be carried out by two distinct enzymatic pathways leading to the production of certain lipid mediators such as, prostanoids via cyclooxygenase or leukotrienes via lipoxygenase. Cyclooxygenases (also known as prostaglandin H synthase or COX) act on arachidonic acid by adding a 15-hydroperoxy group therefore forming PGG_2 and then reducing the hydroperoxy group to the hydroxylated PGH_2 (*9*). There are two cyclooxygenase enzymes involved in the conversion of AA to PGH_2, COX-1 and COX-2. Due to the fact that the 5'-flanking region of the COX-1 gene has few cis-acting response elements and no TATA box, the COX-1 gene has been considered a house keeping gene, although it is not expressed in all tissues or cells (*13*). The COX-2 gene, on the other hand, has several regulatory elements in its 5'-flanking region, including two NF-κB, one Sp1, one NF-IL-6, and one CRE binding site, and is therefore an inducible gene (*13*). COX-2 is also known to be constitutively expressed in the kidney, brain, female reproductive system, and bones, and is important in the normal functioning of these organs (*14*). During an

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inflammatory response IL-1α, IL-1β, TNF-α, interferon-γ (IFN-γ), 12-O-tetradecanoylphorbol-13-acetate (TPA), and lipopolysaccharide (LPS) are inflammatory mediators that induce COX-2 gene expression and subsequent prostaglandin production (*15*).

COX-1 and COX-2 isoenzymes are similar both structurally and mechanistically, both catalyzing a peroxidase and a cyclooxygenase reaction to form PGH_2 (**Figure 1**). These enzymes are present in the luminal surfaces of the endoplasmic reticulum and on both the inner and outer membranes of the nuclear envelope (*13*). When the mature enzymes of COX-1 and COX-2 are produced they contain 576 amino acids and 587 amino acids, respectively (*16*). The differences between amino acid sequences of the two proteins are minor in that a proline residue has been identified in COX-2 after Threonine106 and it contains an eighteen amino acid insertion at its C-terminus, neither of which are present in the COX-1 protein sequence (*16*). At this time the function of these insertions in COX-2 protein are not known, but it is hypothesized that perhaps they may be present to signal subcellular trafficking or protein turnover (*16*). Three structural domains make up the COX enzyme monomer, those being a N-terminal epidermal growth factor like domain (EGF), a membrane binding domain (MBD), and a C-terminal globular catalytic domain that includes a heme binding site that faces the solvent (*17*). It has been suggested that EGF domains act to incorporate the enzyme into the lipid bilayer along with the MBD (*18*).

For both COX-1 and COX-2 enzymes, fatty acid substrates or competing substrates must enter the narrow cyclooxygenase (COX) active site through the MBD directly from the lipid bilayer, suggesting that a conformation change may be necessary to allow for the entry and exit of the substrates (*19*). The active site of COX extends from Arginine120 to Tyrosine385 (*16*). Here, is where the addition of two O_2 molecules to arachidonic acid takes place in the conversion to PGG_2. Arachidonic acid must bind the COX active site in an extended L-shaped conformation, interacting with Arginine120, the known substrate binding site (*20*). This substrate makes 48 van der Waals contacts and two hydrophilic contacts with

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19 residues in the COX active site (*20*). The amino acids deemed important for substrate binding have been identified through site directed mutagenesis. It is known that arachidonic acid may adopt four conformations to bind the COX active site (*21*). The COX active site for COX-2 enzyme has been shown to be larger than that of COX-1 allowing it to accommodate larger substrates without inactivation (*16*).

Opposite the MBD lies the large groove of the peroxidase (POX) active site, which is a long hydrophobic channel with numerous side pockets and a branched water pocket (*16*). The POX active site is relatively open to the solvents, with Histidine207 thought to be important for the deprotonation and subsequent reprotonation that occurs to convert PGG_2 to the alcohol PGH_2 (*22*). Glutamine203 has also been shown to be important during POX catalysis through site directed mutagenesis studies (*22*). After the COX catalytic site has been activated its actions can function independently from the POX cycle, therefore the COX and POX reactions are not tightly coupled (*23*). After the formation of PGH_2 selective synthases act to produce the eicosanoids, such as the prostaglandins.

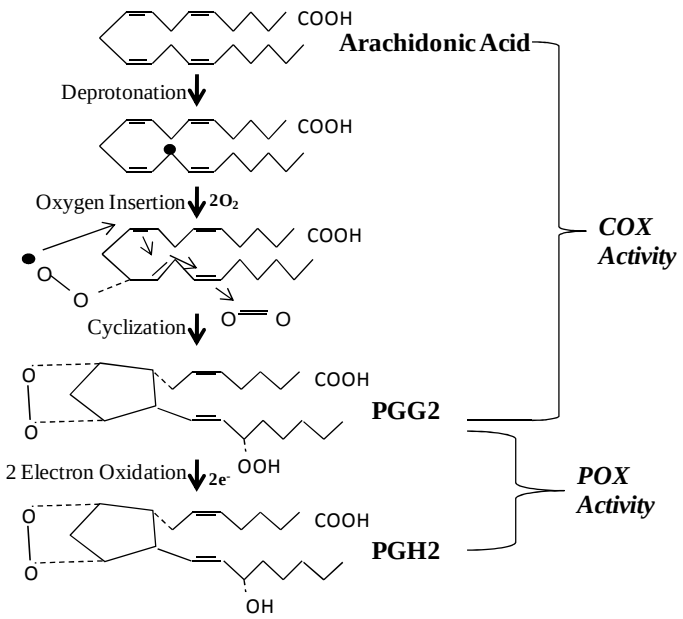

Figure 1. Cyclooxygenase (COX) and peroxidase (POX) activity of COX-1 and COX-2 enzymes

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Prostaglandins (PG) are oxygenated C_{20} fatty acids, which have been shown to be produced in nearly every cell in the body, with PGE_2 being the most abundant prostanoid. Prostaglandins are known to act in an autocrine or paracrine manner to signal changes in the immediate environment (*24*, *25*). Prostanoids formed by the metabolism of AA produce 3 classes of lipid mediators that are involved in a host of different functions (**Figure 2**); including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses. The stable class of prostaglandins is made up of PGE_2, $PGF_{2\alpha}$, PGD_2, and PGJ_2, the labile prostanoids are made up of PG endoperoxides, like PGG_2 and PGH_2, and thromboxane A_2 (TXA_2), and finally the prostacyclin PGI_2 (*26*). The interplay between these lipid metabolites is thought to play a central role in normal human physiology by working to establish homeostasis.

As explained earlier the PG endoperoxides, PGG_2 and PGH_2, are the intermediates that lead to the synthesis of the other eicosanoids, whereas TXA_2 is a highly unstable metabolite that is involved with physiological actions such as platelet aggregation, smooth muscle contraction, and vasoconstriction (*26*, *27*). TXA_2 is prominently produced by platelets in response to various stimuli via the actions of COX followed by thromboxane synthase (TXS) (*28*). Thromboxane A_2 has been implicated in conditions such as asthma, modulation of acquired immunity, atherogenesis, neovascularization, and metastasis of cancer cells through binding to TXA_2 receptor (TP), which is distributed in several areas throughout the body and highly expressed in the thymus and spleen (*27*). Prostacyclin, PGI_2, is formed from the actions of the COX enzymes with further manipulation of PGH_2 by prostacyclin synthase (*26*). Prostacyclin is produced primarily by the endothelium and is able to operate as an antagonist of TXA_2, by preventing platelet formation and clumping involved in blood clotting and acting as a vasodilator (*26*). Together TXA_2 and PGI_2 are considered "specialized" prostaglandins due to their abundance in specific tissues (*14*).

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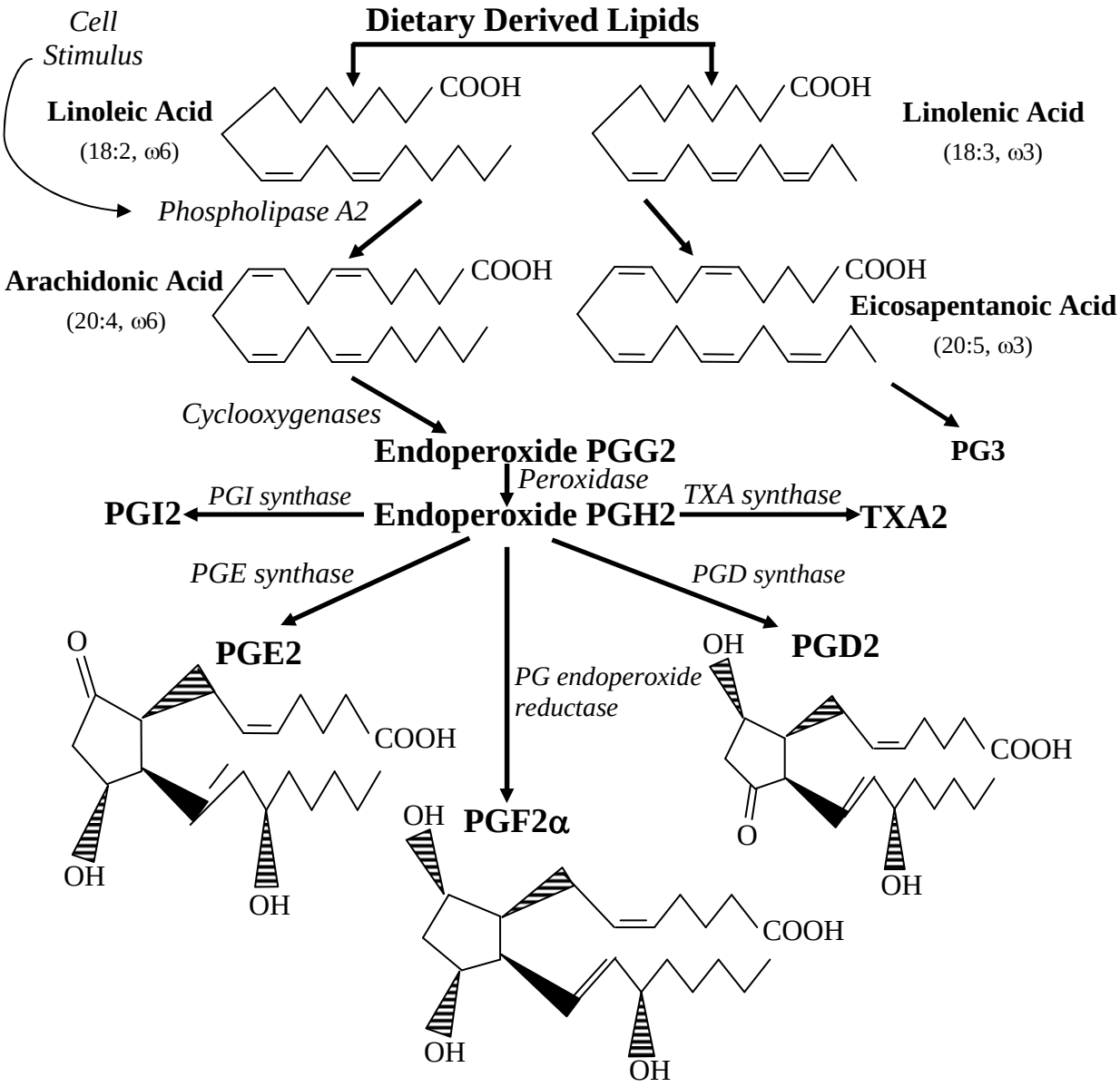

Figure 2. Arachidonic Acid Cascade.

Prostaglandins such as $PGF_{2\alpha}$, PGD_2, and PGE_2 can be synthesized in all cells with the exception of red blood cells and are therefore sometimes referred to as "common" prostaglandins (*14*). The prostaglandins nomenclature is derived from whether it was shown to be soluble in ether (PGE) or phosphate (PGF) buffer (*14*). Prostaglandin F2α is known to

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be the most stable prostaglandin playing a key role in ovarian function, endometrial cyclic changes, embryo development, tubal function, luteal maintenance of pregnancy, induction of labor, childbirth, and more recently has been implicated in inflammation, cardiovascular, and rheumatic diseases (*29*). Another major cyclooxygenase product found in numerous tissues and cell types is $PGD_{2,}$ which is involved in platelet aggregation, relaxation of vascular and nonvascular smooth muscle, and nerve cell function (*30*). Prostaglandin D2 undergoes dehydration to produce prostaglandins of the J variety like PGJ_2, which has been shown to be actively transported to the cell nuclei. In the nuclei, PGJ_2 acts as a potent inducer of cell growth, inhibition and cell differentiation through inhibition of cell cycle progression, suppression of viral replication, induction of heat shock protein expression, and stimulation of osteogenesis (*30*). It has been hypothesized that PGJ_2 is capable of anti-inflammatory activity mediated through peroxisome proliferator-activated receptor-γ, which is a transcription factor present in adipose tissue (playing a central role in adipogenesis), and macrophages (involved in the inhibition of the inflammatory response by inducing apoptosis of activated macrophages) (*31*). Another possible mechanism of the anti-inflammatory effects of PGJ_2 may be through the inhibition of NF-κB activation. Previous reports have indicated that 15d-PGJ_2 inhibits NF-κB activity by binding to the IκB kinase (IKK) or the p65 or p50 transcriptional subunits of NF-κB (*32*).

One of the most intensely studied prostaglandins is prostaglandin E2 (PGE_2), which is a key lipid mediator of inflammation produced by a variety of cells in the body, including fibroblasts, macrophages, and some malignant cell types (*33*). Though PGE_2 is well known for its role in the inflammatory response it also plays an important part in neuronal functions, female reproduction, vascular hypertension, tumorigenesis, fever, gastric mucosal protection, pain hypersensitivity, kidney function, and anti-allergic responses (*10*). Over production of PGE_2 has been associated with various diseases, such as rheumatoid arthritis and even colon

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cancer (*1, 34*). For over 100 years the inhibition of PGE_2 synthesis has been a crucial anti-inflammatory strategy (*35*).

The biological actions of PGE_2 depend upon four types of PGE receptors, EP1, EP2, EP3, and EP4, which are found in various tissues throughout the body and lead to the regulation of intracellular signal transduction pathways (*1*). EP1 receptor is known to stimulate intracellular calcium release, were as both EP2 and EP4 receptors activate adenylate cyclase and induce intracellular cAMP (*36*). The genetic disruption of EP2 in adenomatous polyposis coli (Delta 716) knockout mice, has been linked to a significant reduction in the number of aberrant crypt foci, a precancerous colon cancer lesion, indicating an important role for prostaglandin E2 in oncogenesis (*37*). Also, in female EP2 knockout mice ovulation was shown to be impaired (*38*). Prostaglandin E2 plays many important roles maintaining homeostasis within various parts of the body. For instance, a study examining the role of PGE_2 and eicosanoid receptors in dextran sodium sulfate-induced colitis in mice provided evidence that PGE_2-EP4 signaling was critical in the maintenance of the mucosal barrier and epithelial cell regeneration (*39*). It was demonstrated that disruption of the EP4 gene alone with administration of 3% DSS caused severe colitis and also the addition of PGE_2 to wild type mice with DSS induced colitis completely obliterated symptoms of the disease (*39*). Therefore it is also important to point out that in some instances, with site and receptor specificity, PGE_2 is not always associated with a negative effect.

PGE_2 plays many roles in the body as a potent vasodilator affecting small vessels in the arterioles and is therefore important for the regulation of cardiovascular function (*14*). In the renal system PGE_2 is synthesized in the medulla and is vital for the regulation of salt and water excretion and in the gastrointestinal tract PGE_2 contracts longitudinal muscle and relaxes circular muscle, as well as inhibits gastric secretion (*14*). Prostaglandin E2 has been shown to be a bronchodilator in the respiratory system, balancing the bronchoconstrictor

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functions of PGF_2 (*14*). During bone metabolism PGE_2, produced in the bone has been identified as an important factor stimulating both bone formation and resorption (*14*).

As mentioned earlier, the role PGE_2 plays in immune modulation and therefore inflammation is substantial. It is well known that PGE_2 inhibits T-cell proliferation and has a profound effect on cytokines secreted by T-cells via the induction of T helper 2 (Th2)-type responses (*2*). Prostaglandin E2 has the opposing effect on T helper 1 (Th1)-type cells, and inhibits the production of Th1 cytokines such as interferon γ and IL-2. Other evidence pointing toward the importance of PGE_2 in inflammation has been provided by studies showing that PGE synthase expression is induced by proinflammatory stimuli and during mucosal inflammation T cells are shown to up-regulate the expression of EP receptors (*39*). B cells are also affected by the actions of PGE_2, with PGE_2 suppressing the proliferation of immature B cells or otherwise inducing apoptosis of immature B-cells, without affecting mature B cells. The modulatory affect of PGE_2 can even be seen with "professional" antigen presenting cells, such as dendritic and macrophage cells, where this lipid mediator is partially responsible for the maturation of dendritic cells and the priming of T cells within the lymphoid organs and regulation of cytokine production by activated macrophages. During an inflammatory response, PGE_2 is produced in vast quantities by macrophages in retort to IL-1 and lipopolysaccharide (LPS). Due to the fact that PGE_2 is able to positively regulate its own expression through the up-regulation of COX-2 expression, it is hypothesized that PGE_2 may be an autocrine feedback regulator.

PGE_2 can play a dual role with both inflammatory and anti-inflammatory effects. In activated macrophage cells PGE_2 is able to inhibit TNF-α, IL-6, IL-8, and IL-12 production and up-regulate IL-10 through the EP2 and EP4 receptors, shifting to an anti-inflammatory response (*1*). Eicosanoids derived from AA, such as PGE_2, leukotriene B_4, thromboxane A_2, and 12-hydroxyeicosatetraenoic acid have been identified as present in various cancers, playing key roles in carcinogenesis via modulation of cell cycle responses (*40*). Not only

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does PGE_2 have a profound effect in the inflammatory response but it has been shown to promote tumor cell survival, via inhibition of apoptosis and stimulation of cell proliferation, and was found at greater concentrations in cancer cells than in normal cells (*41*).

There is tight regulation controlling the inflammatory response utilizing both mediators that initiate, sustain, and inhibit inflammation (*42*). When one of these mediators loses control chronic inflammation can result. Chronic inflammation has been linked to a variety of severe diseases, such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, and various cancers (*34*, *43-45*). The impacts of these diseases are astronomical. For instance, 80% of patients with rheumatoid arthritis are disabled after 20 years, with a life expectancy reduced by 3 to 18 years, not to mention the medical expense of such a disease is reported to average $5,919 per case per year in the United States (*43*). In 2005 it was reported that in the United States, approximately one-third of the adult population suffers from arthritis or chronic joint symptoms, that is nearly 70 million people (*46*). Incidence of inflammatory bowel disease has increased dramatically from 1998 to 2004, with a national estimate for total inpatient charges for Crohn's disease and ulcerative colitis increasing from $762 million to $1,330 million and $592 million to $945 million, respectively (*47*).

The battle against inflammatory diseases with symptoms such as pain and inflammation has been fought by two major classes of pharmaceuticals, those being non-steroidal anti-inflammatory drugs (NSAIDS) or cyclooxygenase-2 specific inhibitors (COX-2 inhibitors). Back in 1893, the German chemist Felix Hoffman produced a molecule with analgesic activity called acetylsalicylic acid or aspirin, giving rise to a new class of drugs called NSAIDs (*25*). In 2002, over 41 million prescriptions were filled in the United States for COX-2 inhibitors and the total prescription costs of NSAIDS were estimated at $6.5 billion in 2003 (*48*). Synthetic NSAIDs were designed to inhibit the activity of both cyclooxygenase isoforms, COX-1 and COX-2, in order to decrease the formation of

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prostaglandins, which are responsible for inflammation, swelling, pain, and fever (*49*). These drugs also showed promise for cancer prevention, because they were shown to reduce the risk of certain cancers. Over time it became clear that long term NSAID use caused gastrointestinal (GI) and renal toxicity, leading to peptic ulcer formation and increase of ulcer hemorrhage (*50, 51*). There have been well designed studies matching NSAID users against controls showing NSAIDs to increase serious GI events by 1.5 to 7.2 fold (*52, 53*).

Although it is now considered a naïve notion, it was once believed that COX-1 was the housekeeping enzyme responsible for maintenance of intestinal mucosal integrity and producer of basal levels of prostaglandins, which protected the gastric mucosa and it was therefore the inhibition of this isoform that was responsible for the notorious side effects observed in those who used NSAIDs (*49*). It was hypothesized that in order to get rid of the severe GI side effects only one of the cyclooxygenase isoforms, COX-2, should be targeted for inhibition, as it was thought to be the more inducible enzyme after exposure to mitogens or inflammatory stimuli. Selective COX-2 inhibitors were developed in the 1990s, originally for arthritis symptoms, but were later used in clinical trials for the treatment of colorectal cancers (*46*).

Celecoxib was the first selective COX-2 inhibitor to be introduced into the pharmaceutical market in December of 1999, other synthetic inhibitors followed by the names of Rofecoxib (Vioxx), Valdecoxib, and Lumiracoxib (*46*). The Vioxx Gastrointestinal Outcomes Research (VIGOR) study demonstrated a reduced incidence of GI lesions after long-term use of rofecoxib, compared to a popular NSAID called naproxen (*54*). In September of 2004, Merck and Co withdrew Vioxx from the market, due to data from the VIGOR study and another internal study providing evidence that the drug increased risk of heart related problems (*46, 54*). Although COX-2 is indeed inducible, it is also constitutively expressed in the glomeruli of the kidney and the cortex of the brain and the inhibition of this enzyme could help explain the reports of heart attack and stroke that are associated with long

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term COX-2 inhibitor use (*46*). It has been hypothesized that perhaps COX-2 is important in protecting cardiomyocytes from oxidative injury or that COX-2 dependent PGI_2 production interacts with inducible nitric oxide synthase to increase the heart's tolerance to future ischemic insults but further studies are required to pinpoint the mechanisms leading to these events (*49*). Until researchers are able to develop or identify a natural or synthetic pharmaceutical capable of providing the benefit of relief from inflammatory disease, without the risk of GI or cardiac problems, patients must weigh the risk versus the benefit of taking such drugs and consume accordingly.

Signal Transduction Pathways Important for Inflammation

Nuclear factor-κB (NF-κB) is a transcription factor involved in the transcription of proinflammatory and antiapoptotic genes, including COX-2 and iNOS (*55*). It is known that NF-κB is a key player in the progression of cancer, rheumatoid arthritis, inflammatory bowel disease, and asthma (*56*). When the classical (canonical) NF-κB pathway is not stimulated, the pathway is inhibited by the association of inhibitor of NF-κB (IκB) with the Rel dimers that make up the NF-κB transcription factor. IκB does not allow the Rel dimers to translocate into the nucleus and activate transcription of target genes (*57*). The NF-κB canonical pathway can be stimulated by inflammatory cytokines (including TNF-α, LPS, growth factors, stress inducers, chemotherapeutic agents, etc.). Upon stimulation of the NF-κB pathway, the high molecular weight inhibitor of NF-κB kinase (IKK), made up of three subunits IKKα, IKKβ, and IKKγ, is activated leading to the phosphorylation of four serine residues on the two subunits of the inhibitor of NF-κB kinase (IkB). These phosphorylations lead to the ubiquitination and degradation of IκB by 26S proteosome (*57*). The uninhibited NF-κB Rel subunits are then able to translocate into the nucleus of a cell and carry out transcription of target genes.

An alternative or non-canonical pathway of NF-κB has been shown to occur in B cells, which appears to be responsible for the development, survival, and attenuation of

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apoptosis of B cells (*58*). This alternative pathway can be stimulated by lymphotoxin (LT) β receptor, B cell activating factor (BAFF) receptor, or CD40, as opposed to the TNF-α receptor known to activate the classical pathway (*58*). During activation of the alternative NF-κB pathway processing of precursor protein p100 to p52 occurs, through the stimulation of NF-κB binding kinase (NIK) and IKKα resulting in the phosphorylation of p100. The phosphorylated precursor protein, p100, then undergoes controlled proteolysis by the proteasome producing the mature protein, p52, which is then able to form dimers with RelB and activate transcription of target genes necessary for secondary lymphoid organogenesis, mature B cell function, and adaptive immunity (*58*).

An important facet to the activation of the NF-κB response relies heavily on which dimers bind and act on the NF-κB consensus sequence. There are five members of the NF-κB transcription factor family that were discovered in the 1980s, p105 (constitutively processed to p50), p100 (processed to p52), p65 (also known as RelA), RelB, and c-Rel, each subunit having different functional domains (*59*). All five members contain a Rel-homology domain (RHD) allowing them to bind to DNA, but only p65, RelB, and c-Rel contain transactivation domains (TADs) which sets the stage for which combinations of transcription factors are activating or repressive (*60*). For example, one of the most common heterodimers of NF-κB consists of p50 and p65, which is able to activate transcription because TAD is present in p65, whereas a homodimer of p50 would be repressive because no TAD exists.

Genetic knockouts have provided extensive information on the importance of each transcription factor on health and inflammation. Mice with genetic deficiencies in genes that encode p50, p52, c-Rel, and Rel B have abnormal responses from immune cells dealing with B and T cell proliferation, antigen presentation, isotype switching, and cytokine production, although these mice appear healthy and develop normally (*60*). It has also been demonstrated that knockouts for p65, IKKβ, and IKKγ die in late embryonic development or at the time of birth due to TNF-α regulated hepatocyte apoptosis (*61*). Similar knockouts of

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IKK subunits have been studied for their role in inflammatory diseases resulting in chronic inflammation in intestinal epithelial cells, showing a massive influx of macrophage cells expressing TNF-α (*62*). This state was normalized by crossing the IKK knockouts with TNF receptor knockout mice, indicating a fragile equilibrium between the inflammatory response and apoptosis (*60*).

Mitogen-activated protein (MAP) kinases are important serine/threonine kinases that play a major role in genetic signaling pathways crucial for the control of embryogenesis, cell differentiation, cell proliferation, and apoptosis (*63*). There exists four known subgroups within the MAP kinase family, Extracellular signal-regulated kinases (ERK), c-jun N-terminal or stress activated protein kinases (JNK/SAPK), ERK5/bigMAP kinase1 (BMK1), and p38 protein kinases. p38 MAPK is the primary kinase from this family that is involved in inflammation, consisting of four homologs, p38α and β, which are isoforms of each other that are ubiquitously expressed, and p38δ and γ, which are differentially expressed. The gene expression of p38δ has been observed in the lung, kidney, testis, pancreas, and small intestine, whereas p38γ expression is predominantly located in skeletal muscle (*64*). Several extracellular stimuli are known to activate the p38 MAP kinase pathway, including microbial products, such as lipopolysaccharide (LPS), and a number of cytokines such as IL-1α, IL-2, IL-7, IL-17, IL-18, TGF-β, and TNF-α. After the activation of this signaling cascade p38 MAPK is known to up-regulate the expression of many genes involved in the inflammatory response, like TNF-α, IL-1β, IL-6, IL-8, COX-2, collagenase-1, -3.

Echinacea

The genus *Echinacea* (Purple Cone Flower) is a perennial flower native to the central United States. Its name comes from the Greek word *echinos*, which means sea urchin or hedgehog, referring to the spiked floral receptacle (*65*). There are nine species of *Echinacea*: *E. purpurea*, *E. angustifolia*, *E. pallida*, *E. tennesseensis*, *E. sanguinea*, *E. simulata*, *E. laevigata*, *E. atrorubens*, and *E. paradoxa*, each with moderate to extensive variations in

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their phytochemical profile (*66*). *Echinacea* has been used medicinally for several hundred years, dating back to Native American peoples use of this herb for various ailments, including pain relief for coughs and sore throats, fevers, smallpox, mumps, measles, rheumatism, arthritis, and antidotes for poisons and other venoms (*67*). *Echinacea angustifolia* was the most popular species used by the Native Americans, though records show that they also used *E. purpurea*, and *E. pallida* (*67*). The first published account of the medicinal use of *E. purpurea* was in 1762, in the 2nd edition of Gronovius' Flora Virginicus, where Laurens Theodoor Gronovius reported its beneficial use for saddle sores on horses (*65*). *Echinacea* has a long history of its use medicinally and yet still warrants further study and understanding of its potential therapeutic uses.

Currently *Echinacea* is most commonly used to treat the common cold and other upper respiratory tract infections (URTI). Formulations of this botanical supplement can be found in the form of salves, tinctures, capsules, teas, injections, etc. (*68*). In the United States annual sales of *Echinacea* products have been estimated to range from more than $200 million to more than $300 million (*69*). With such large scale purchase and therefore use of this botanical supplement there are still questions of its efficacy in modulating the immune system and its mechanisms of action, as well as what constituents are important for various bioactivities. Clinical trials investigating the usefulness of *Echinacea* in treating colds and URTIs have provided inconsistent results and will be the topic of further review in following sections. A possible reason for the lack of congruity with *Echinacea* studies may be due to the preparations used in the studies. The quality of plant material and commercial products available to consumers has been examined both in the United States and abroad. In an analysis of 59 commercial products available in the United States and labeled as *Echinacea*, it was discovered that 10% had no detectable *Echinacea* content, 48% were inconsistent with their label when identifying the species present, and of 21 standardized preparations, 57% did not meet the standards declared on their labels (*70*).

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Major Bioactive Constituents Identified in *Echinacea* Species

The five major classes of compounds that are thought to be responsible for the bioactive properties identified in *Echinacea* extracts are alkylamides, caffeic acid derivatives, polyacetylenes (generically known as ketones), polysaccharides, and glycoproteins (*65*). Each species of *Echinacea* contains different, yet in some cases overlapping, chemical profiles that allow for their identification. Structures of common alkylamides, ketones, and caffeic acid derivatives identified as present in the *Echinacea* species are drawn in **Figure 3**, along with the proper nomenclature for these constituents.

Echinacea Alkylamides

Twenty-five alkylamides, also known as alkamides, have been identified in *Echinacea* roots and aerial parts (*65*), and of these, fourteen have been found in the roots of *E. angustifolia* and eleven have been found in the roots and aerial portion of *E. purpurea* (*71*) The two major alkylamides found in *E. angustifolia* and *E. purpurea*, are dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8 and 9) and are known to occur as a mixture (*72*). Total alkylamide concentrations have been reported to range from 0.004% to 0.039% dry weight (*65*). When chewing on *Echinacea* roots or seeds a tongue tingling or numbing sensation is experienced due to the alkylamide constituents (*65*). It has been hypothesized that alkylamides are beneficial to the *Echinacea* plant by acting as insecticides. Clifford et al. conducted a mosquitocidal assay showing that the mixture of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (Bauer alkylamides 8/9) caused 87.5% mortality of mosquito larvae within 15 minutes at a concentration of 405 μM (*73*). Other research conducted on alkylamides isolated from arabidopsis indicates a possible role for this constituent in promoting plant growth and root development (*74*). With many hypotheses circulating on how alkylamides may be critical to the *Echinacea* plant, others are

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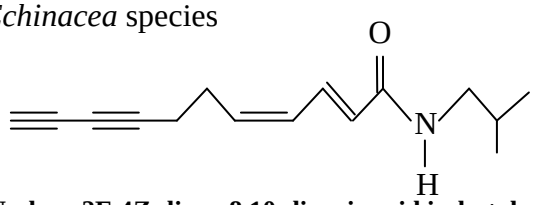
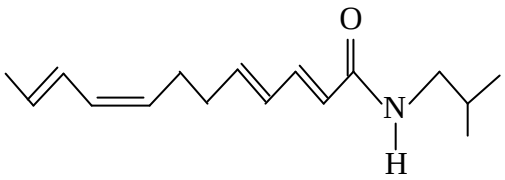
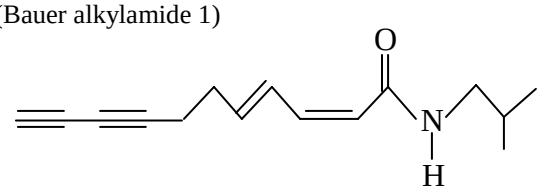
Figure 3. Structures of common alkylamides, ketones, and caffeic acid derivatives found in *Echinacea* species



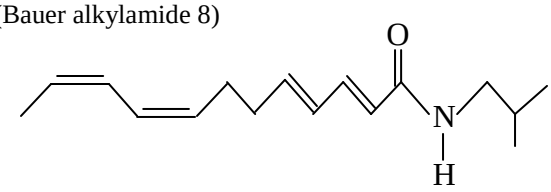
Undeca-2E,4Z-diene-8,10-dienoic acid isobutylamide (Bauer alkylamide 1)



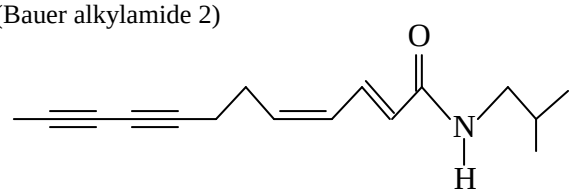
Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide (Bauer alkylamide 8)



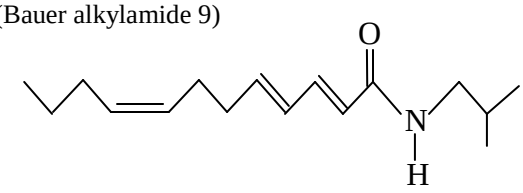
Undeca-2Z,4E-diene-8,10-dienoic acid isobutylamide (Bauer alkylamide 2)



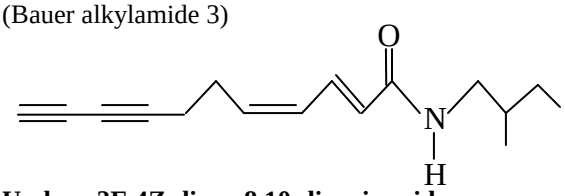
Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Bauer alkylamide 9)



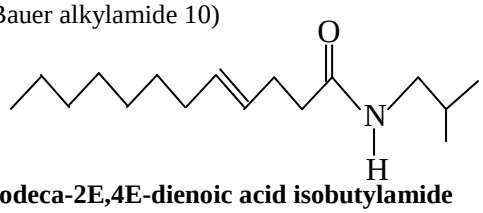
Dodeca-2E,4Z-diene-8,10-dienoic acid isobutylamide (Bauer alkylamide 3)



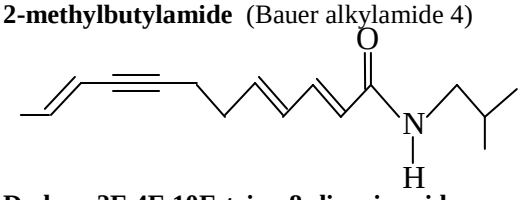
Dodeca-2E,4E,8Z-trienoic acid isobutylamide (Bauer alkylamide 10)



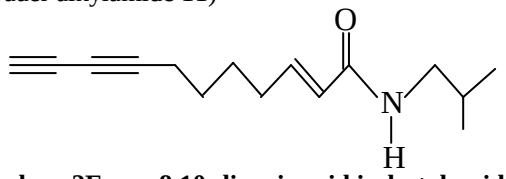
Undeca-2E,4Z-diene-8,10-dienoic acid 2-methylbutylamide (Bauer alkylamide 4)



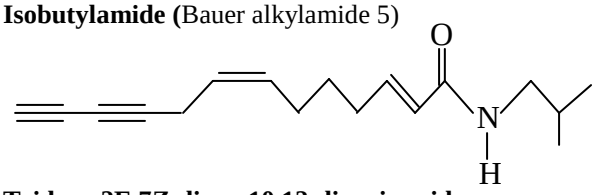
Dodeca-2E,4E-dienoic acid isobutylamide (Bauer alkylamide 11)



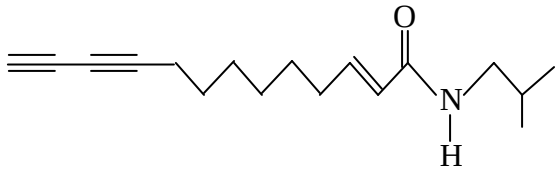
Dodeca-2E,4E,10E-trien-8-dienoic acid Isobutylamide (Bauer alkylamide 5)



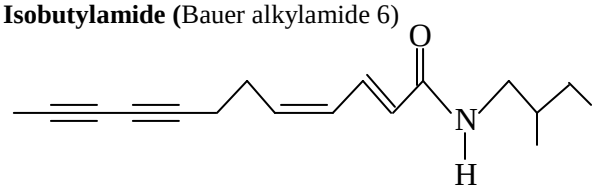
Undeca-2E-ene-8,10-dienoic acid isobutylamide (Bauer alkylamide 12)



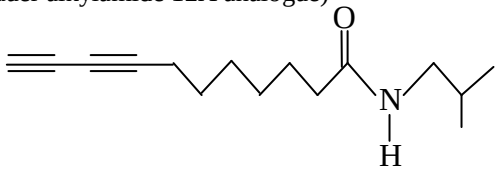
Trideca-2E,7Z-diene-10,12-dienoic acid Isobutylamide (Bauer alkylamide 6)



Trideca-10,12-dienoic acid isobutylamide (Bauer alkylamide 12A analogue)



Dodeca-2E,4Z-diene-8,10-dienoic acid 2-methylbutylamide (Bauer alkylamide 7)



2,3dihydro-Undeca-2E-ene-8,10-dienoic acid isobutylamide (Bauer alkylamide 12B analogue)

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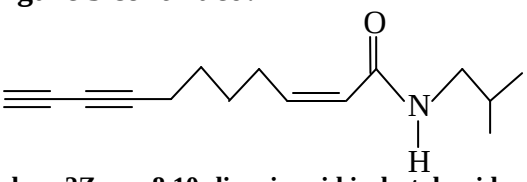
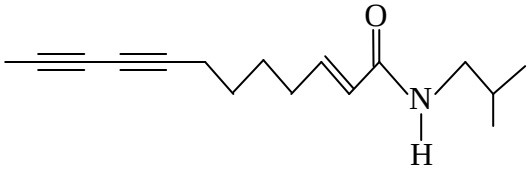
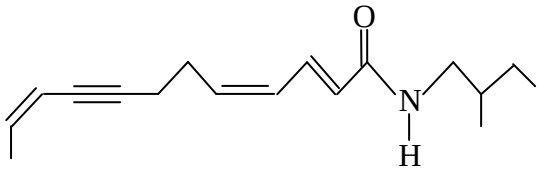
Figure 3 continued.



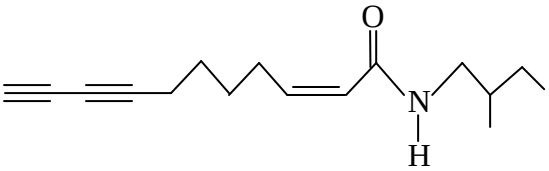
Undeca-2Z-ene-8,10-dienoic acid isobutylamide
(Bauer alkylamide 13)



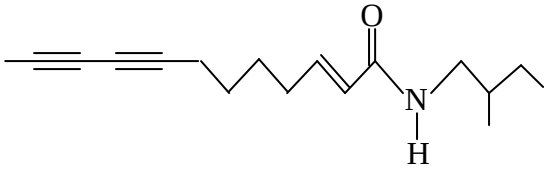
Dodeca-2E-ene-8,10-dienoic acid isobutylamide
(Bauer alkylamide 14)



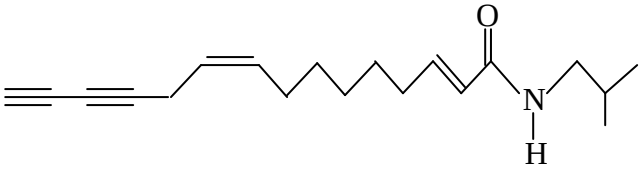
Dodeca-2E,4Z,10Z-trien-8-dienoic acid isobutylamide
(Bauer alkylamide 15)



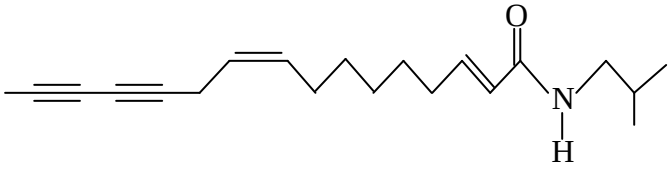
Undeca-2Z-ene-8,10-dienoic acid methylbutylamide
(Bauer alkylamide 16)



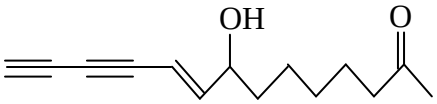
Dodeca-2E-ene-8,10-dienoic acid 2-methylbutylamide
(Bauer alkylamide 17)



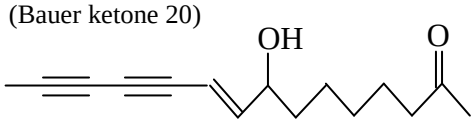
Pentadeca-2E,9Z-diene-12,14-dienoic acid isobutylamide
(Bauer alkylamide 18)



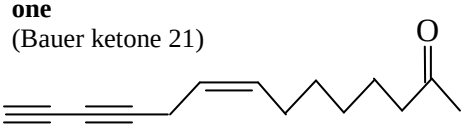
Hexadeca-2E,9Z-diene-12,14-dienoic acid isobutylamide
(Bauer alkamide 19)



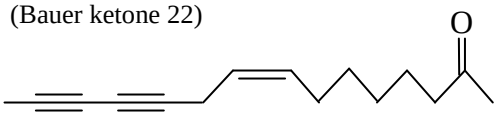
8-hydroxytetradeca-9E-ene-11,13-diyn-2-one
(Bauer ketone 20)



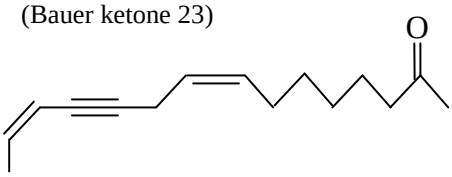
8-hydroxypentadeca-9E-ene-11,13-diyn-2-one
(Bauer ketone 21)



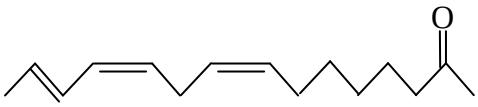
Tetradeca-8Z-ene-11,13-diyn-2-one
(Bauer ketone 22)



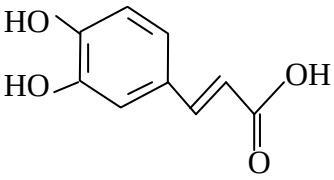
Pentadeca-8Z-ene-11,13-diyn-2-one
(Bauer ketone 23)



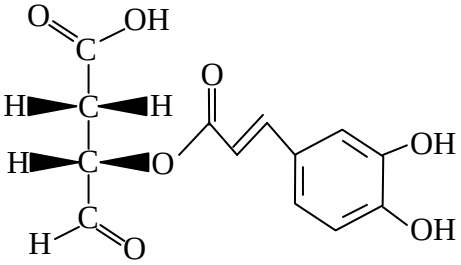
Pentadeca-8Z-ene-11,13-diyn-2-one
(Bauer ketone 24)



Pentadeca-8Z,11Z,13E-trien-2-one
(Bauer ketone 25)



Caffeic Acid



2-O-caffeoyl tartaric acid
(Caftaric acid)

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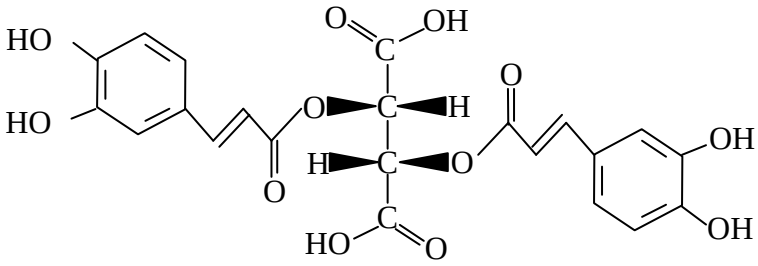
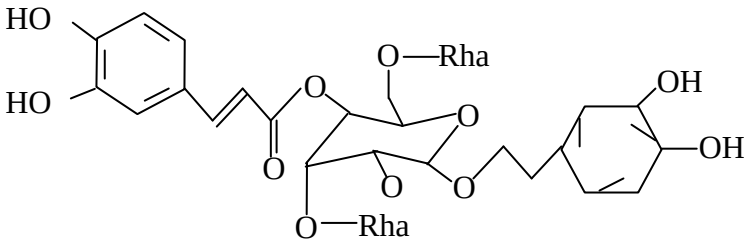
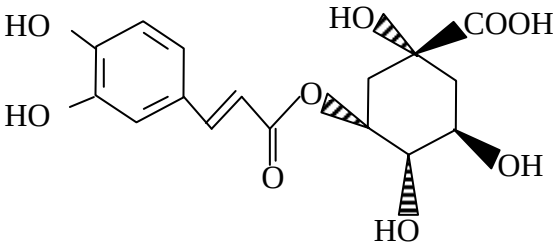
Figure 3 Continued.



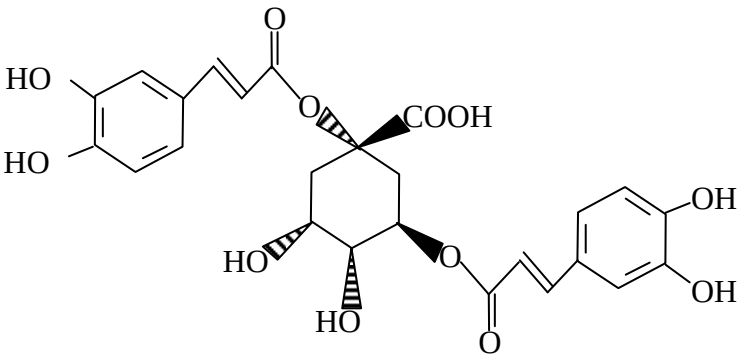
2,3-O-dicaffeoyl tartaric acid
(Cichoric acid)



Echinacoside



3-O-caffeoyl quinicic acid
(Chlorogenic acid)



1,3-O-dicaffeoyl quinic acid
(Caftaric acid)

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interested in alkylamides found in *Echinacea* due to their immune modulating capabilities in human, animal, and cell culture studies, as well as studies that are providing evidence that these constituents are bioavailable (*75, 76*).

The inhibition of COX-2 has been of great interest to those studying inflammation and several studies set out to show that alkylamides from *Echinacea* have such inhibitory capabilities. Early studies published in 1994 examined eight alkylamides isolated from an n-hexane extract of *E. angustifolia* for their ability to reduce production of prostaglandin E2 and 5-hydroxyeicosatetraenoic acid as a measure of cyclooxygenase and 5-lipoxygenase activities, respectively. The *E. angustifolia* extract was shown to inhibit both activities at concentrations of 50 μg/ml for cyclooxygenase and 11.5 μg/ml for lipoxygenase, with an inhibition of 62.4% and 81.8%, respectively (*77*). When the alkylamides were screened it was shown that a 1:1 mixture of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8/9) inhibited both cyclooxygenase by 54.7% at a concentration of 50 μM and 5-lipoxygenase by 62.2% at a concentration of 50 μM (*78*). In another study focusing on cyclooxygenase activity, alkylamides isolated from the roots of *E. purpurea*, undeca-2Z,4E-dien-8,10-diynoic acid isobutylamide (Bauer alkylamide 2), undeca-2E,4Z-dien-8,10-diynoic acid 2-methylbutylamide (Bauer alkylamide 4), and dodeca-2E,4Z-dien-8,10-diynoic acid 2-methylbutylamide (Bauer alkylamide 7) were shown to inhibit COX-1 activity in an *in vitro* model from microsomal preparations of ram seminal vesicles, with inhibitions of 60%, 55%, and 48%, respectively (*73*). COX-2 activity was also inhibited by Bauer alkylamides 2, 4, and 7 with inhibitions of 46%, 39% and 31%, respectively (*73*). The concentration of alkylamide used to inhibit cyclooxygenase activity was actually quite high in the previously described study at 100 μg/ml, which is equivalent to 437 μM, 412 μM, and 389 μM, for Bauer alkylamides 2, 4, and 7, respectively. Inhibition of cyclooxygenase-2 dependent PGE_2 was identified in neuroglioma cells (H4 cells) treated with alkylamides isolated from a CO_2 extract made from the root material of *E. angustifolia* (*78*). Only the *E.*

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angustifolia extract and 10 μM of undeca-2Z-ene-8,10-diynoic acid isobutylamide (Bauer alkylamide13), dodeca-2E-ene-8,10-diynoic aid isobutylamide (Bauer alkylamide 14), and dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide (Bauer alkylamide 7) were able to significantly inhibit PGE_2 of the eight alkylamides screened (*78*). Further analysis of COX-2 protein and mRNA, showed an increase of COX-2 at the transcriptional and translational level, but a significant suppression of COX-2 enzyme activity with treatment of Bauer alkylamides 13, 14, and 7 (*78*). The insight that was gained from the studies described above provided a basis for the hypothesis that perhaps alkylamides act by competing with arachidonic acid for the cyclooxygenase enzymes and that may be how PGE_2 is attenuated, though more relevant studies are necessary.

Another means by which alkylamides have been shown to modulate the immune system and act in an anti-inflammatory nature, through the activation of macrophage cells, is by inhibiting the production of nitric oxide (NO). Chen et al. conducted a study to determine if 14 alkylamides from the roots of *E. angustifolia*, *E. purpurea*, and *E. pallida* could reduce the amount of NO produced after stimulation of RAW264.7 mouse macrophage cells with lipopolysaccharide (*79*). The lowest NO ID_{50} was determined to be 12 μM for both dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide (Bauer alkylamide 3) and dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide (Bauer alkylamide 7), with dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (Bauer alkylamides 8/9), dodeca-2E,4E-dienoic acid isobutylamide (Bauer alkylamide 11), and dodeco-2E,4Z,10Z-trien-8-ynoic acid isobutylamide (Bauer alkylamide 15) coming in next with a ID_{50} of ~24 μM (*79*). Cell viability was also measured using an MTT assay after treatment of RAW264.7 cells with alkylamides, showing TD_{50} at doses ≥50 μM with all alkylamides screened (*79*). High levels of nitric oxide production (via iNOS and eNOS) plays an important role in the inflammatory response through increasing vascular permeability, angiogenesis, and the production of free radicals, such as peroxynitrites, which lead to tissue damage. The importance of identifying

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constituents in *Echinacea* that act in a multifaceted fashion during the process of inflammation, through the inhibition of multiple inflammatory endpoints, is essential to understanding the full potential of this herbal product.

Alkylamides Proposed Mechanism Leading to Immune Modulation

Little is understood about the molecular mechanism of action *Echinacea* employs to exert its immunomodulatory properties, but studies have been accumulating providing insight to this topic. In 2004, Gertsch et al. published a study demonstrating that dodeca-2E,4E,8Z-10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8/9) induce TNF-α mRNA, as well as cAMP, p38/MAPK, and JNK signaling, and activate NF-κB in primary human monocytes/macrophages, but do not inhibit LPS induced TNF-α protein levels at nanomolar concentrations (*80*). It was postulated that the mechanism leading to the described immune modulation was related to cannabinoid receptors, providing the first insight into a possible mechanism of action for *Echinacea* (*80*).

Cannabinoid receptors (CB1 and CB2) are G protein coupled receptors that have been shown to potentially modulate a variety of immune cell functions in various species including humans (*81*). The CB1 receptor is ubiquitously expressed in various regions of the brain and is partially responsible for catalepsy, depression of motor activity, analgesia, feelings of relaxation, and well being (*82*). It should also be mentioned that CB1 receptor is highly expressed on monocytes, macrophages, dendritic cells, and osteoclasts (*83*). Whereas the CB2 receptor is located in the periphery and is known be present in immune cells, such as T cells, B cells, natural killer cells, macrophages, neutrophils, and mast cells (*82*).

Anandamide (C:22) is a derivative of arachidonic acid and an endogenous ligand for the cannabinoid receptors (*84*). Due to the fact that alkylamides share structural similarity with anandamide, Woelkart et al. assessed alkylamides isolated from *E. angustifolia* root for their ability to bind to rodent cannabinoid receptors using a receptor binding assay, as well as the metabolic stability of these constituents to fatty acid amidohydrolase (FAAH) (*85*).

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FAAH is responsible for the hydrolysis and inactivation of anandamide and therefore may be involved in metabolism of alkylamides (*86*). Bauer alkylamides 8/9, 10, 11, and 17 showed the greatest affinity for rat CB1 receptors with Ki values ranging from 6.7 μM to 11.0 μM, but when phenylmethanesulfonyl fluoride was added as an inhibitor of FAAH there was a slight decrease in the Ki concentrations for Bauer alkylamides 10, 11, and 17, suggesting that alkylamides are also susceptible to hydrolysis by serine-dependent proteases (*85*). As for the CB2 receptor, Bauer alkylamides 7, 8/9, 10, 11, and 17 and tetradeca-2E-ene-10,12-diynoic acid isobutylamide showed the greatest affinity with Ki values ranging from 1.9 μM to 12.7μM (*85*). There is particular interest in cannabinoid receptor CB2 ligands due to their potential to act as anti-inflammatory agents, without interfering with the detrimental brain function effects linked to CB1.

Further studies on alkylamides and their ability to bind to human CB2 receptor determined that dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Bauer alkylamide 9) and dodeca-2E,4E-dienoic acid isobutylamide (Bauer alkylamide 11) bound to the receptor more strongly than endogenous ligands, such as Δ^9-tetrahydrocannabinol, anandamide, and 2-AG (*81*). The Ki values of Bauer alkylamides 9 and 11 were ~60 nM for CB2, with significantly less affinity for the CB1 receptor (*81*). It was previously shown by Gertsch et al. that alkylamides influence cellular cAMP levels in human monocytes/macrophages and that they inhibit induced cAMP production, providing a basis to study whether alkylamides binding to CB2-positive or CB2-negtive cell lines effect total [Ca^{2+}] (*80, 81*). In the CB2-positive cell line, Bauer alkylamides 9 and 11 induced [Ca^{2+}], with no significant modulation of [Ca^{2+}] in CB2-negative cells, indicating that CB2 is important for Ca^{2+} signaling with alkylamides (*81*).

Macrophage cells have been identified as crucial cells involved in the inflammatory response and therefore scientists are interested in understanding how *Echinacea* alkylamides or caffeic acid derivatives may influence biomarkers, such as NF-κB expression, TNF-α

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production and nitric oxide production, which are known to be inducible in macrophage cells. A recent study described how certain classes of alkylamides affect these endpoints differently in the RAW264.7 mouse macrophage cell line (*87*). It was determined that after treatment with LPS, cichoric acid at 8 ng/ml, a mixture of alkylamides from an *Echinacea* extract (EPL AA) at 2ng/ml, and (2E, 4E, 8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (Bauer alkylamide 11) at 2 ng/ml, which represents the 2,4-diene class of alkylamides, were shown to inhibit NF-κB reporter gene expression, but only cichoric acid at 0.8ng/ml and the mixture of alkylamides in an *Echinacea* fraction 2 ng/ml were able to decrease TNFα production (*87*). Finally, only the mixture of alkylamides from the *Echinacea* fraction at 2 µ/ml were shown to inhibit NO production (*87*).

A similar study conducted by the same group that provided the macrophage study examined the effects of alkylamides on human T cells in an *in vitro* model system that utilized Jurkat cells (*88*). T cells are another group of immune cells derived from the thymus that play an important role in both activating other cells, such as B cells and macrophages during an inflammatory response, or differentiating into cytotoxic T cells that are capable of killing virally infected cells (*2*). The study was designed to observe the effect of two classes of alkylamides, 2-ene and 2,4-diene, and caffeic acid on the modulation of NF-κB expression in a luciferace model system (*88*). NF-κB is a nuclear transcription factor which can be stimulated during an inflammatory response to express genes like TNFα, IL-1, and COX-2. It was determined that (2E)-N-isobutylundeca-2-ene-8,10-diynamide (Bauer alkylamide 12) at 2 µg/ml, which represents the 2-ene class of alkylamides was able to inhibit NF-κB gene expression after treatment with LPS and (2E,4E,8Z,10Z)-N-isobutyldodeca-2,4,8,10-tetraenamide (Bauer alkylamide 11) at 0.2 µg/ml, which represents the 2,4-diene class of alkylamides was shown to significantly increase NF-κB reporter gene expression after LPS treatment. When phorbol myristate acetate was treated prior to addition of alkylamide or caffeic acid derivative, both cichoric acid at 8 µ/ml and Bauer alkylamide 11at 2 µg/ml

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significantly increased NF-κB gene expression, whereas Bauer alkylamide 12 at 0.2 µg/ml was shown to significantly decrease NF-κB luciferase expression (*88*). Such a differing effect between 2-ene and 2, 4-diene alkylamides seen in the studies of RAW264.7 mouse macrophage cells and human T cells reviewed above should caution investigators of *Echinacea* to the importance of understanding which constituents are crucial for a particular bioactivity.

Anti-viral Activity of *Echinacea* Alkylamides

Other studies with alkylamides have provided evidence toward their dual action as both anti-inflammatory agents, as well as having an indirect anti-viral effect. Interleukin-2 is required for the clonal expansion and activation of T cells during infection and has also been linked to decreased symptoms and reduction of virus revival in experimental rhinovirus infection (*2, 89*). It has been hypothesized that modulatory effects on IL-2 protein production may manipulate T cell specific immunity toward infection (*90*). Sesagawa et al. set out to identify whether aerial *E. purpurea* extracts or constituents could modulate phytohemagglutinin/phorbol 12-myristate 13-acetate induced IL-2 production in Jurkat E6.1 T cells (*90*). From this study it was determined that aerial extract of *E. purpurea* in 95:5 ethanol/water inhibited IL-2 production by ~65% at 50 µg/ml and two standard alkylamides, dodeca-2E, 4E, 8Z,10Z-tetraenoic acid isobutylamide (Bauer alkylamide 9) and dodeca-2E,4E-dienoic acid isobutylamide (Bauer alkylamide 11) at concentrations of 0.625 µg/ml to 25 µg/ml (equivalent to that in the *E. purpurea* extract), were both capable of dose-dependent significant IL-2 suppression compared to a vehicle control, with caffeic acid derivatives showing no significant alteration of IL-2 production at concentrations present in the *E. purpurea* extract (*90*).

Echinacea Caffeic Acid Derivatives

Caffeic acid derivatives make up a major class of constituents found in *Echinacea*, that include, caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, and cichoric

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acid. Pellati et al. reports the total phenolic content for *E. angustifolia*, *E. pallida*, and *E. purpurea* to be 10.49 mg/g, 17.83 mg/g, and 23.23 mg/g, respectively (*91*). This class of compounds has been studied for anti-oxidant capabilities through measuring their ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radicals. Six caffeic acid derivatives were screened with the order of scavenging potency against $DPPH^{\bullet}$ radicals as follows: echinacoside (EC_{50} = 6.6 μM) > cichoric acid (EC_{50} = 8.6 μM) > cynarin (EC_{50} = 11 μM) > chlorogenic acid (EC_{50} = 18.9 μM) > caffeic acid (EC_{50} = 19.1 μM) > caftaric acid (EC_{50} = 20.5 μM) (*91*). These results are consistent with previous reports indicating that the ability of phenolic compounds to scavenge free radicals is largely influenced by the number of hydroxyl groups on the aromatic ring, with more hydroxyl groups having greater activity (*92*).

Caffeic acid derivatives have been studied for their anti-viral properties. L-chicoric acid has been reported to potently inhibit human immunodeficiency virus type 1 (HIV-1) integrase activity, which is a key enzyme in the viral life cycle required for replication, in a HeLa CD4 LTR-β-gal cell line model transfected with HIV-1 (*93*). Furthermore, it was shown that synthetic dicaffeoylquinic acids and dicaffeoyltartaric acids are potent and selective inhibitors of HIV-1 integrase and replication (*94*). The antimutagenic potential of caffeic acid derivatives found in *Echinacea* (L) *Moench* was analyzed using the *salmonella typhimurium* tester strain TA100 in a plate-incorporation test, where revertants were counted (*95*). The results demonstrated that cichoric acid actually increased the mutagenicity of 3-(5-nitro-2-furyl)acrylic acid (5NFAA), whereas ferulic and caffeic acids were identified to be antimutagenic by decreasing the mutagenic effect of 5NFAA by approximately 50%, although it should be noted that extremely high concentrations of 300 to 5000 μg/ml were screened to identify this effect (*95*).

Stability of Alkylamides and Caffeic Acid Derivatives

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A major concern of researchers studying the bioactivity of the constituents found in *Echinacea*, as well as those using and preparing *Echinacea* treatments for medicinal applications, is the stability of active constituents after various preparations, storage temperatures, and storage periods. It has been hypothesized that due to the known anti-oxidant bioactivity of the caffeic acid derivatives that perhaps they are protective of the highly unsaturated alkylamides via preventing oxidation. Several studies have set out to understand the ideal conditions of preparation and storage for *Echinacea* products and will be discussed below.

During 1999, Livesey et al. used either an alcoholic *Echinacea purpurea* three year old root extract or a dried powder from that same extract to examine the stability of marker compounds (Bauer alkylamide 1/2 and a phenolic, cichoric acid) after storage at -20°C, 25°C, or 40°C for seven months. After the seven month period HPLC was used to determine that in the dried powder, alkylamide concentration decreased 88% at 25°C and 95% at 40°C, whereas the cichoric acid concentration was not significantly affected (*96*). A reverse effect was identified for the liquid extract where the alkylamide level remained stable over the temperature range and cichoric acid content declined 80% at the higher temperatures (*96*). Another study looked at the effect of chopping, drying, and storage (both time and temperature) of *E. purpurea* roots and how those conditions affected alkylamide content. It was determined that drying had no effect, whereas chopping did affect certain alkylamides slightly but to a non-significant degree. The major findings were that alkylamides levels decreased by greater than 80% during storage at 24°C after a 64 week period (*97*). *Echinacea* roots stored at -18°C also experienced a significant decline in alkylamide levels after the same storage period, indicating that attention must be paid to storage temperatures. These were some of the earlier results to indicate that an optimization of preparation and storage conditions was necessary to maintain stability of two main constituents found in *Echinacea*.

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A common method that is commercially used to dry harvested raw material from *Echinacea* is that where hot air is fan-forced over plant material. In 2003, Stuart and Wills designed a study to examine the alkylamide and cichoric acid concentration of *E. purpurea* root and aerial portions dried in the fan-forced manner at temperatures ranging from 40-70°C (*98*). Alkylamide content did not differ at the assorted drying temperatures in the root, stem, or leaf material, but there was a significantly higher amount of alkylamide in flowers dried at 70°C as compared to those dried at 40°C (*98*). The alkylamide data collected indicates great stability for these constituents at drying temperatures ranging from 40-70°C. Overall, as the drying temperature increased, cichoric acid content significantly decreased in root, flower, stem, and leaf materials (*98*).

Other studies sought to determine whether long term storage of *Echinacea* material had an effect on bioactivity. In 2006, Senchina et al. determined that greater than one year storage of dry *Echinacea* roots from seven of the nine species maintained their cytokine-modulating capabilities in an *in vitro* model of Type A influenza vaccination (*99*). Another study examined the immunomodulatory effects of seven *Echinacea* species tinctures after 2 years of storage at -20°C. Two sets of experiments were employed, one with human peripheral blood mononuclear cells (PMBC) treated with the *Echinacea* tinctures only and the other with PMBC collected from vaccinated individuals immunized with influenza vaccine and then treated with *Echinacea* tinctures and influenza virus. Results indicated an increased alkylamide concentration for *E. purpurea*, *E. simulata*, and *E. tennesseensis*, with no change identified for *E. angustifolia*, *E. pallida*, or *E. sanguinea* after 2 years of storage at -20°C (*100*). Another significant finding was that cytokine production was measured to be higher with fresh extracts than the stored extracts providing evidence that storage time may be detrimental to obtaining consistent results in bioassays (*100*).

In 2007, Liu and Murphy determined that there were differences in the stability of alkylamides stored in solution versus dry extract and that phenolics, such as the caffeic acid

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derivatives are likely to play a role in protecting alkylamides from degradation. Four different accelerated storage conditions were used to prepare *E. purpurea* extracts, those being, phenolic-depleted and phenolic-rich dry and phenolic depleted and phenolic-rich dissolved in dimethyl sulfoxide (DMSO) (*101*). Extracts were stored under 70°C, 80°C, and 90°C. The results from this study showed that dry *E. purpurea* alkylamides degraded significantly slower with phenolic acids present than without, but yet the alkylamides degraded faster in phenolic-rich DMSO extracts than those without phenolics (*101*). A key finding from this study was that DMSO extracts maintained the stability of the alkylamides better than the dry extracts (*101*). The authors concluded that different mechanisms must account for alkylamide oxidation in solution compared to the dry extracts.

From the studies discussed in this section it can be concluded that several factors, such as preparation method, temperature during preparation, temperature during storage, and storage duration are very important for the biological evaluation of constituents found in medicinal herbs. Also, because of the medium chained structure of alkylamides, its double bond contents, and unsaturated configuration this constituent is highly prone to oxidation, which perhaps under certain conditions is circumvented through the actions of radical scavenging caffeic acid derivatives (*101*). These findings should make researchers mindful of the need to control for stable conditions of constituents of interest and to be diligent to conduct research in a timely manner to obtain meaningful results before degradation has the opportunity to occur.

Echinacea Ketones

Acetylenes, generically called ketones, are the major lipophilic compounds found in certain accessions of *Echinacea pallida* and until recently have not been studied as thoroughly as other constituents for their bioactive potential. A study focusing on the cytotoxic properties of five ketones found in n-hexane extracts of *E. pallida* determined that pentadeca-(8Z,13Z)dien-11-yn-2-one (Bauer ketone 24) was able to cause a concentration

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dependent decrease in cultured human colorectal (COLO320) and pancreatic (MIA PaCa-2) adenocarcinoma cell viability, with an IC_{50} of 2.34±0.33 μM and 32.17±3.98 μM, respectively (*75*). These results were quite significant because the study goes on to show that Bauer ketone 24 was more effective then the common cancer drug 5-flourouracil at decreasing cell viability in COLO320 cells and that this ketone causes cytotoxicity through activation of caspase 3/7 (*75*). Another study analyzed the cytotoxic properties of Bauer ketone 24 on leukemia (Jurkat and HL-60), breast carcinoma (MCF-7), and melanoma (MeWo) cells, identifying significant cytotoxic properties with IC_{50} less than 2.5 μM for the Jurkat and MCF-7 cells (*102*). The cytotoxic effect of the *Echinacea* ketone was determined to be due to cell cycle arrest during the G_1 phase in the HL-60 leukemia cells (*102*).

Further research has been conducted to better understand whether constituents found in *Echinacea* are able to influence 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 *Echinacea pallida* extract that pentadeca-(8Z,13Z)dien-11-yn-2-one (Bauer ketone 24) was able to decrease the efflux of the Pgp probe calcein-AM by three-fold compared to the control at a concentration of 30 μM (*103*). Although the inhibition measured with Bauer ketone 24 was minimal compared to that of other Pgp inhibitors it is interesting to consider the roles this inhibition may take in herb-drug interactions.

In 2008 an attempt was made to determine a possible mechanism of action for reported immunomodulatory properties of ketones found in *E. pallida* (*104*). Egger et al. chemically synthesized natural and non-natural ketoalkenes from *E. pallida* and tested for antagonistic activation via the cannabinoid receptors using a GTPase assay (*104*). No ketoalkenes were capable of activation of GTPase activity to greater than 20% of basal levels and therefore were not considered cannabinoid receptor agonists (*104*). Further studies will

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need to be completed to determine a mechanistic basis for the bioactivities associated with ketones from *Echinacea*.

A stability study was conducted with pentadeca-(8Z, 13Z)-dien-11-yn-2-ome (Bauer ketone 24) in cell culture medium (Roswell Park Memorial Institute 1640 medium supplemented with L-glutamine, fetal bovine serum, penicillin, and streptomycin) over a 72 hour period under standard culture incubation conditions (*102*). Results from this study indicated that oxidation of the ketone was relatively slow, with the area of hydroperoxide intermediate at approximately 25% at 72 hours and the final hydroxylated ketone derivative was not detected after 72 hours (*102*).

Echinacea Polysaccharides

Polysaccharides are constituents found in *Echinacea* extracts that have been recognized to possess macrophage activating bioactivity, including the ability to induce macrophages to develop high cytotoxicity against tumor cells. It is noteworthy to point out that polysaccharides are extracted from *Echinacea* using water and therefore parallel screens for endotoxin levels should be performed in conjunction with the identification of bioactivities, to ensure that observed results are a direct consequence of the polysaccharide treatment and not due to increased endotoxin stimulation. Endotoxin studies were not mentioned in the studies described hereafter. In 1984 studies were conducted utilizing purified polysaccharides from *E. purpurea* extracts targeting immune cells both *in vitro* and *in vivo* that provided evidence that this class of compounds possess macrophage-activating properties, in both peritoneal macrophages and macrophages cultivated from bone marrow precursor cells (*105*). It was shown that the polysaccharides activated the macrophages to develop extracellular cytotoxicity toward DBA/2 P815 mastocytoma cells, indicating a possible application for this group of constituents in combating tumor systems (*105*).

Luettig et al. further explored polysaccharides and macrophage activation by isolating highly purified acidic arabinogalactan from cell cultures of *E. purpurea* and published further

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evidence that thioglycollate-induced peritoneal macrophages were stimulated to produce TNF-α, bone marrow macrophages were stimulated to secrete IFN-beta, and resident peritoneal macrophages were stimulated to produce IL-1 when treated with arabinogalactan (*106*). This same study explained that arabinogalactan activated macrophages to kill TNF-α sensitive WEHI164 tumor cells and increase phagocytosis of the parasite *Leishmania enrietti* (*106*). Another study that verified these results was described by Steinmuller et al. demonstrating that polysaccharides separated from *E. purpurea* cell cultures were able to activate peritoneal macrophages in animals after administration of cyclophosphamide or cyclosporin A, known immunosuppressors, by increasing the production of TNF-α, as well as increase cytotoxicity against WEHI164 fibrosarcoma tumor cells and an intracellular parasite *Leishmania enrietti* (*107*). Evidence is accumulating suggesting that arabinogalactan and other polysaccharides may exert therapeutic effects against certain cancer cells as well as various parasites, due to their cytotoxic properties.

Melchart et al. conducted a clinical study utilizing a polysaccharide fraction isolated from an *E. purpurea* extract to neutralize the adverse side effects experienced by individuals having chemotherapy treatments for advanced gastric cancer (*108*). Thirteen patients received daily i.v. injections of 2 mg of a polysaccharide fraction for 10 days, beginning 3 days before initiating their chemotherapy regimen (*108*). Results were very unclear for this study due to the control group coming from historical records of past patients with gastric carcinoma that were treated with the same chemotherapy agents (*108*). The study at most suggested a slight yet non-significant decrease in leukopenia, which is a decrease in circulating white blood cells that leaves the patient overly susceptible to infection, in the patients that received the polysaccharide fraction (*108*).

Echinacea Glycoproteins

Other studies discuss the possible role glycoproteins may have in the immunostimulating effects observed with *E. purpurea* extracts through the activation of

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complement. Mechanistic studies have shown that arabinogalacatin protein type II (AGP) isolated from the pressed juice of *E. purpurea* binds to C1q, can enhance C3-consumption, and further induce an increase of C5b-9, which is the membrane attack complex, thereby stimulating potent complement activation (*109*). Alban et al. verified these results using a haemolytic complement assay, providing evidence that AGP was able to activate both the classical and alternative pathways of complement (*110*).

Classen et al. studied the proliferative effect of different concentrations of AGPs on mouse spleen cells, describing that AGPs isolated from *E. pallida* roots dose-dependently activated proliferation, whereas AGPs purified from suspension culture of *E. purpurea* were ineffective proliferators (*111*). Further results demonstrated that APGs isolated from both *E. pallida* and *E. purpurea* increased IgM-titres in spleen cell cultures from NMRI mice, indicating an ability to activate B-lymphocytes (*111*). When alveolar mouse macrophages were treated with *E. pallida* and *E. purpurea* AGPs, a dose-dependent increase was observed in IL-6 production and nitrite production, which is an expected outcome of macrophage activation (*111*).

The Anti-viral Activity of *Echinacea* Preparations

In western countries the common cold is one of the most prevalent diseases with direct medical costs reported to reach approximately $16.8 billion annually (*112*). In the U.S. an adult suffers from 2 to 4 colds per year, accounting for approximately 23 million days of work absence (*112*). With the vaccination for rhinoviruses remaining elusive and clinical benefits from zinc, vitamin C, and other antihistamines unclear, there is a need to identify other sources for treatment of these viruses that cause the symptoms associated with the common cold (*113*).

Echinacea has been touted for its use to prevent or treat upper respiratory tract infections (URI), as well as the common cold. Although several prevention trials, self-treatment, and treatment trials have been conducted to examine the efficacy of *Echinacea*

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products in preventing or treating URIs and cold symptoms, a clear answer still eludes researchers. There have been a mixture of positive and negative results, due to several different experimental variables, including time to initiation of treatment, virus type and dose, immune competence of volunteers, and proper characterization of the *Echinacea* products being studied (*12, 114*). Due to the variability associated with the results from *Echinacea* clinical trials, meta-analyses are being used to pool results from well designed studies looking for a common endpoint, that being the efficacy of *Echinacea* extracts to prevent and limit the duration of symptomatic development of a naturally induced or an experimentally induced cold.

In a meta-analysis reported by Schoop et al. in 2006, 231 of 234 articles were eliminated because they were used to study spontaneous common colds, leaving 3 high quality, double blind, placebo controlled, adequately randomized studies to be pooled for the analysis of *Echinacea's* efficacy in treating the common cold after clinical inoculation (*113*). Two studies were conducted by Turner et al. in 2000 and then in 2005, utilizing different species with each study, with the earlier *E. purpurea* extracts and the later *E. angustifolia* extracts (*115, 116*). Sperber et al. provided the third study included in the Schoop et al. meta-analysis, treating subjects with *E. purpurea* extracts (*117*). In the meta-analysis of these three studies, the data for the *Echinacea* extracts were pooled and compared with placebo, providing evidence for prophylactic efficacy (*113*). It was determined that the probability of developing a clinical cold was 55% higher in the placebo control group than those that received *Echinacea* as a treatment (*113*). Another meta-analysis was conducted in 2007 by Shah et al. that included trials that were randomized placebo-controlled, used to evaluate *Echinacea*-containing products (extracts containing *E. purpurea*, *E. angustifolia*, *E. pallida*, or a combination of species), measuring prevention and/or treatment of the common cold, and contained adequate data on either cold incidence or duration (*118*). After inclusion parameters were considered, 14 studies were chosen for meta-analysis, providing evidence

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that *Echinacea* decreased the odds of contracting a cold by 58% and decreased the duration of cold by 1.4 days (*118*). It is important to note that the Shah et al. meta-analysis pooled trials regardless of the product tested and incorporated trials even when *Echinacea* preparations were combined with other herbal products (*113*). Both meta-analyses discussed here point toward a beneficial outcome when considering the efficacy of *Echinacea* products to prevent or treat the common cold, yet there is still a need to conduct larger prevention trials in order to arrive at a distinct conclusion.

The clinical studies along with the meta-analysis described in the previous section have provided a strong basis to identify *Echinacea* as having anti-viral properties. A micro-array study looking at which genes are differentially expressed when human bronchial epithelial cells either infected with human rhinovirus type 14 (RV14) or uninfected and treated with an aqueous polysaccharide-enriched extract from the aerial portion of *E. purpurea* (E1) or an ethanolic tincture from the roots of *E. purpurea* (E2) found several affected genes lie in the immune response category (*119*). From this study it was determined that E1 and E2 increased expression of certain cytokines like MIP-1α, IL-8, and TNF-α, but decrease the expression of IL-6, IL-1β, and IL-10 (*119*). When RV14 viral infection increased the expression of two genes known to be involved in the innate immune response, mycovirus resistance gene MS1 and IFNα-induced protein 15, both E1 and E2 reversed the viral stimulation (*119*).

Anti-oxidant Properties of *Echinacea* Extracts

Echinacea extracts have also been reported to be free radical scavengers and possess anti-oxidant properties. In 2004, Sloley et al. showed that the roots of *E. purpurea* exhibited significantly greater free radical scavenging capabilities than either *E. angustifolia* or *E. pallida* based on a model measuring the accumulation of 2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical (*120*). That same study also determined that root or leaf extracts of *E. purpurea*, *E. angustifolia*, and *E. pallida* inhibited Fe^{2+}-induced lipid peroxidation (*120*).

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Echinacea and Immune Modulation

Echinacea extracts have been touted as having immunomodulatory properties, yet most of the time in the literature, only ethanol extracts are assayed. Pillai et al. used flow cytometry to determine which type of extract, EtOH or water, was able to stimulate CD4+ cells to the greatest extent (*121*). Mature T helper cells express the glycoprotein CD4+ which can be used as an indicator of immune activation (*2*). It was determined that only the aqueous extracts from *E. purpurea* were significantly active, stimulating CD4+ cells by 24 % at a concentration of 100 μg/ml, whereas EtOH material was inactive even at very high doses, though it is important to point out that endotoxin levels were not measured in this experiment, which may be higher in the water extract (*121*). From this study the authors suggest that it is the water soluble (polysaccharides) that are responsible for the immunostimulatory activity found in the *Echinacea* species (*121*). More studies are warranted to make conclusions about a certain group of constituents accounting for immunomodulatory properties, based on the evidence presented in the Pillai et al. study only measured one endpoint.

Another study focusing on an aqueous extract of *E. purpurea* determined through flow cytometric methods that this extract at 0.01 μg/ml was able to activate natural killer cells, indicated by the expression of CD69 and down regulation of CD16, increase NK-target cell binding, and increase killer cell frequency in human peripheral blood mononuclear cells (*122*). These results again suggest a role of *Echinacea* polysaccharides in NK maturation and possibly support the idea that *Echinacea* has anti-viral properties.

Regulation of Dietary Supplements Including *Echinacea*

One of the greatest misconceptions that has developed out of public opinion on herbal products has been that these products are natural, (being from the environment) so they are therefore safe to use medicinally. This common belief among consumers may be due to the fact that dietary supplements, including botanical supplements, are not regulated directly by

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the U.S. Food and Drug Administration (FDA) in the same manner as synthetic pharmaceuticals.

The 1994 Dietary Supplement Health and Education Act (DSHEA) allowed Americans to enjoy access to vitamins, minerals, and herbal products. DSHEA requires manufacturers to study and identify the uses of the products labeled under this act and provide the FDA with adequate safety information 75 days prior to marketing their product. DSHEA also sets provisions as to how these supplements can be labeled which allows the manufacturer to make structure/function or nutritional support claims that must be truthful and indicate the role of the nutrient in supporting wellness or heath. Another labeling requirement brought about by this act states that the dietary supplement label must list the name and quantity of each active ingredient, identify the product as a dietary supplement, and if the supplement is an herb the manufacturer must identify the part of the plant from which it is taken. Unfortunately, there has been misleading information identified from studies analyzing herbal content on manufacturer's labels. For example, in Germany a study was conducted to analyze alkylamide and cichoric acid content in commercial root preparations of *E. purpurea* showing that out of 25 preparations some products were highly concentrated, whereas others neither class of constituent was detectable, identifying excessive variability in chemical profile amongst various preparation methods labeled as *Echinacea* (*123*). The results from this study indicate that it is necessary to quantitate potentially bioactive constituents, especially in preparation for clinical trials examining efficacy of this herb in the prevention of cold symptoms, etc.

In the United States 25 commercial preparations of *Echinacea* were also assessed for labeling accuracy, finding that only 56 % passed the assessments for their quality. Some of the products were flawed in that they were improperly labeled, contained a different species than what was present on the label, contained excessive amounts of microbes, or contained no trace of constituents claimed on the label (*124*). These studies provide evidence to the

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consumer that not all manufacturers are strictly following the guidelines and regulations placed by DSHEA. The consumer must realize that just because something is marketed as a natural product does not mean that it is safe. Although few adverse side effects have been reported with the use of *Echinacea*, the fact of the matter is that research is still under way to determine the safety and efficacy of this herbal product. Consumers must continue to be leery and identify which dietary supplement products have the potential to be beneficial for their health and weigh the risk of potential side effects, interactions with other drugs, or cost.

DSHEA also established the Office of Dietary Supplements (ODS) within the National Institutes of Health to identify the role of dietary supplements in improving health and encouraging scientific research that seeks to establish the benefits of dietary supplements dealing with the prevention of or cures for disease (http://www.fda.gov/opacom/laws/dshea.html). Through the diligent work by ODS, researchers studying dietary supplements, and mainstream media becoming more educated on topics of herbal supplements, scientists and consumers alike are developing a better understanding of the safety and efficacy of many of these products being marketed today.

Adverse Effects Associated with *Echinacea*

Echinacea products are among the most commonly self prescribed herbal remedies selected today, with approximately 16% of patients undergoing chemotherapy using them to supplement cancer therapy (*125*). A recent study focused on the interference of fractions and pure compounds segregated from *E. angustifolia* roots, with doxorubicin, a common anticancer drug known to be cytotoxic against several cancer cell lines (*125*). Doxorubicin was treated to cervical and breast cancer cells at two doses (0.05 μM and 0.5 μM), either in the absence or presence of *E. angustifolia* fraction, cynarine, chicoric acid, or echinacoside (*125*). This study follows up a previous study which discovered that *Echinacea* has the ability to interact with anticancer drugs oxidized by the cytochrome p450 isoform, 3A4 (*126*). Cervical cancer HeLa cells were shown to increase in cell growth after treatments

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with cynarine by 48-125 % or 29-100 % with simultaneous treatments of 0.05 μM and 0.5 μM doxorubicin, respectively (*125*). Chicoric acid was able to significantly increase cell growth of the HeLa cells by 23-100 % when also treated with 0.05 μM doxorubicin (*125*). Studies conducted with the MCF-7 breast cancer cells did not identify a significant interaction between doxorubicin and the caffeic acid derivatives, but did determine that the ethyl acetate fraction increased cell growth by 20-25 % (*125*). It can be concluded from this study that certain compounds and fractions isolated from *E. angustifolia* have growth promoting activity in certain cancer cells, which actually can adversely affect the cytotoxic activity of the common chemotherapy drug doxorubicin. Interactions with herbal supplements need to be more thoroughly examined in order to protect the consumer.

It is becoming increasingly more popular for atopic individuals to use complementary alternative medicines (CAM) and is therefore not surprising that allergic responses to CAM are quite common in these people (*127*). An Australian study evaluated 5 patients who suffered adverse reactions to *Echinacea* exposure, including anaphylaxis, acute asthma, mild asthma, and rash (*128*). The patients were subjected to skin prick testing (SPT), including a commercially available glycerinated extract of *Echinacea*, where three of the five had positive SPT results. *Echinacea* belongs to the Asteraceae family, which consists of greater than 20,000 species worldwide and therefore exposure to members of this family is inevitable throughout a lifetime, with sensitization to Asteraceae being quite common (*127*). The 2004 Australian study also examined fifty-one adverse drug reports indicating that *Echinacea* as the culprit, but determined that only 26 of these cases were suggestive of IgE mediated hypersensitivity (*128*). The study concluded that atopic people were over represented in the group reporting adverse consequences from *Echinacea* and that it was possible that other environmental allergens cross-react with *Echinacea* to set off the reported allergic reactions. From this data it should be noted that atopic individuals should be leery when consuming any botanical.

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A single case of *Echinacea* induced cholestatic autoimmune hepatitis has been reported in the medical literature based on a case with a 45-year-old male, who was admitted into hospital care complaining of fatigue and jaundice for a duration of 1 week (*129*). He had revealed that he took 1500 mg/day of *Echinacea* root after catching a cold the previous month (*129*). The liver biopsy demonstrated interface hepatitis, obvious cholestasis, and portal lymphoplamocytic and eosinophilic granulocyte infiltration. One month after discontinuing use of the *Echinacea* supplementation the patient had elevated liver enzymes, positive IgG, and spontaneous normalization of laboratory values (*129*).

Bioavailability and metabolism of constituents found in *Echinacea*

Bioavailability can be defined as the portion of a particular dose of constituent that arrives at the systemic circulation as an intact constituent (*130*). A major weakness with most studies dealing with botanicals and bioactivity is accounting for how much of the particular extract or compound actually enters the cells or the body of the model being studied. Within the past seven years more and more studies are focusing on the bioavailability of constituents found in *Echinacea*. Alkylamides, caffeic acid derivatives, and more recently ketones found in *Echinacea* have been studied for their bioavailability through a popular model of the intestinal epithelial barrier called the Caco-2 monolayer system (*76, 131*).

An early study conducted in 2002 examined the bioavailability of dodeca-2E,4E,8Z,10E/Z-tetra-enoic acid isobutylamides (Bauer alkylamides 8/9) through the Caco-2 cells over a 6 hour time period (*131*). Results from this study indicated that after loading the apical side of the monolayers with 25 μg/ml of Bauer alkylamides 8/9, approximately 15% of these constituents were detectable within 30 minutes on the basolateral side, with similar results recorded when the adenocarcinoma colonic cell line was pretreated with lipopolysaccharide or phorbol 12-myristate-13-acetate in order to mimic an inflammatory state (*131*). It was also noted that no significant metabolism had occurred, because

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approximately 80% of alkylamides 8/9 were recovered on the basolateral side after the 6 hour incubation. In a separate experiment using this model system twelve alkylamides were reported to readily cross from the apical to the basolateral side of the monolayers (apparent permeability $>10^{-6}$ cm/s), with this process taking only 90 minutes for over half of the compounds (*76*). This study also showed that 2,4-dienes were more readily transported than the equivalent 2-ene alkylamides (*76*). Within the 90 minute time period the caffeic acid derivatives, such as, cichoric acid, caftaric acid, cinnamic acid, echinacoside, and tartaric acid exhibited poor uptake through the Caco-2 monolayers, with less than 5% transported (*76*). Chicca et al. also used the Caco-2 monolayer *in vitro* assay to provide evidence that 8-hydroxy-pentadeca-(9E,13Z)-dien-11-yn-2-one, pentadeca-(8Z,13Z)dien-11-yn-2-one (Bauer ketone 24), and tetradeca-(8Z)-ene-11,13-diyn-2-one (Bauer ketone 22) were readily permeable through the cell system, with Bauer ketone 22 showing the highest apparent permeability at $32\pm3 \times 10^{-6}$ cm/s (*75*). These studies indicate that alkylamides, as well as certain ketones, are likely to cross the intestinal barrier and therefore should be able to carry out their bioactivities *in vivo* and that caffeic acid derivatives are less likely to do so. As previously discussed it has been shown that Bauer ketone 24 at 30 µM was able to inhibit P-glycoprotein (Pgp) drug transport in a HK-2 cell line model, which would have important implications on drug interactions (*103*). A more recent study published in 2008 identified that an ethanol extract of *E. purpurea* was able to significantly decrease the basal to apical transfer of digoxin at concentrations ranging from 0.4 to 6.36 mg/ml in a Caco-2 monolayer cell system (*132*). These results provide evidence that high concentrations of *E. purpurea* are capable of a dose dependent inhibition of Pgp which may have limited importance to drug bioavailability.

Alkylamides have been the focus of research dealing with constituents found in *Echinacea* due to the various, previously mentioned bioactivities these constituents possess. It is no surprise that alkylamides have also been the focus of clinical trials looking at

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bioavailability of *Echinacea*. *In vitro* models have suggested that alkylamides are bioavailable and clinical trials that have been conducted are mounting stronger evidence that this is indeed the case (*76, 130, 133, 134*). Dietz et al. conducted preliminary studies to optimize a technique to measure alkylamide content in human blood (*135*). Blood was collected from 4 humans on regular diets and spiked with four dilutions of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8/9) to create a calibration curve and subjected to HPLC, having recovery rates greater than 95 % (*135*). One healthy human subject consumed an *E. purpurea* tincture containing 4.3 mg Bauer alkylamides 8/9, which was shown to be detectable by HPLC and quantified to 44 ng/ml blood, providing the basis for more clinical work (*135*).

In 2005, two single dose human clinical trials were published, again providing evidence that alkylamides are bioavailable. Matthias et al. examined plasma samples from 9 healthy subjects that had ingested tablets manufactured from EtOH root extracts of *E. angustifolia* and *E. purpurea* after consuming a high fat breakfast, reporting rapid absorption of alkylamides (sum of alkylamides averaging 336±131 ng eq/ml plasma) that were detectable 20 minutes after the tablet was taken (*133*). This study also reported that 130 mg of caffeic acid derivatives were also ingested from the tablets, yet none were detectable in any of the plasma samples, coinciding with the previous Caco-2 monolayer work (*133*). Woelkart et al. recruited 11 volunteers to fast overnight and consume an oral dose of 2.5 ml of 60% EtOH root extract of *E. angustifolia*. Undeca-2E/Z-ene-8,10-diynoic acid isobutylamides (Bauer alkylamides12/13) were the first to reach their maximum plasma concentration of 1.87 ng/ml at 20.1 minutes and dodeca-2E,4E,8Z, 10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8/9) showing the greatest maximum plasma concentration of 10.88 ng/ml after 30.3 minutes, indicating that alkylamides can be quickly absorbed in rather high amounts with certain structures (*135*).

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Because *Echinacea* is used in many formulations throughout the world, another clinical trial examined the bioavailability of 3 different preparations of *E. purpurea* in the form of Echinaforce™ tablets, Echinaforce™ Junior tablets, and *Echinacea* sore throat spray, as well as the pharmacokinetics of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (Bauer alkylamides 8/9) after ingestion (*130*). In this cross-over study, after an overnight fast volunteers were orally administered either 12 Echinaforce™ tablets containing 143.2 μg of Bauer alkylamides 8/9, 10 Echinaforce™ Junior tablets containing141.3 μg of Bauer alkylamides 8/9, or 4.4 ml of *Echinacea* sore throat spray, and plasma was collected at five time points after dosing (*130*). Bauer alkylamides 8/9 were shown to be bioavailable in all formulations distributed to the human volunteers although they appeared at different time points (ranging from 28 minutes with the Echinaforce™ tablets to 68 minutes with the Echinaforce™ Junior tablets), but with similar C_{max} ranging from 0.22 – 0.23 ng/ml (*130*).

From these clinical studies it is becoming more evident that alkylamides are constituents that should be capable of eliciting their pharmacological effects *in vivo*, but other studies examining how constituents are metabolized by the gut microflora may suggest a need to examine other aspects of the physiology involved in bioavailability besides the presence of these constituents in blood plasma.

Due to potential interactions with other herbal supplements or pharmaceuticals, it is necessary to understand how alkylamides from *Echinacea* are metabolized in the liver or in other tissues. In 2005, Matthias et al., studied the metabolism of the alkylamide constituents from *Echinacea* premium liquid, in human liver microsomes and provided evidence that NADPH dependent cytochrome p450 metabolized 2-ene and 2,4-dienes differently (*136*). When cofactor-, enzyme-, and time-dependent degradation of substrates were analyzed as a measure of metabolism it was shown that synthetic 2,4-dienes were quickly metabolized with only 9% and 2% of the original concentration remaining of Bauer alkylamides 9 and 11, respectively (*136*). A number of metabolites from Bauer alkylamide 9 were identified with

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LCMS, showing peaks with apparent molecular weights that would indicate epoxidation, hydroxylation, and N-dealkylation (*136*).

In a follow-up study, Jurkat human T cells were used to assess the influence of *Echinacea* alkylamides and metabolites on cytokine production after previous incubation with human liver microsomes (*137*). It was previously shown that alkylamide metabolism by cytochrome p450 was dependent on the presence of NADPH and thus alkylamides were incubated in human liver microsome in the presence of NADPH and major metabolites were detected, including a novel carboxylic acid metabolite (dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, also known as Bauer alkylamides 8/9) by LC-MS. It was determined that incubation times around 120 minutes of Bauer alkylamides 8/9 and NADPH with human liver microsomes favored the formation of the carboxylic acid metabolite. Further results from this study showed that an *E. purpurea* extract containing 4 μg/ml of Bauer alkylamides 8/9 significantly repressed IL-2 secretion by 47% in the Jurkat T cells, but when NADH was present with the same treatment the significant suppressive effect on IL-2 secretion was lessened, indicating the importance of understanding the metabolism of constituents found in *Echinacea* and how it affects different bioactivities (*137*).

Hypothesis and objective

One long-term goal of the Center for Research on Dietary Botanical Supplements is to optimize and enhance the production of anti-inflammatory constituents present in *Echinacea* to make this botanical an effective supplement for the relief of inflammatory symptoms. The goal of this particular research project was to identify *Echinacea* extracts, fractions, and alkylamides capable of inhibiting prostaglandin E2 (PGE_2), and elucidate the signaling targets involved in this anti-inflammatory contribution, as well as identify which constituents found in *Echinacea* are of greatest importance for PGE_2 inhibition. The central hypothesis for this research stated that *Echinacea* extracts, fractions, and alkylamides inhibit the NF-kB signal transduction pathway, which in turn inhibits the expression of

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cyclooxygenase-2 and further inhibits the production of prostaglandin E2 in RAW264.7 mouse macrophage cells, with alkylamides playing a key role in this anti-inflammatory response.

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CHAPTER 3: *ECHINACEA* SPECIES AND ALKYLAMIDES INHIBIT PROSTAGLANDIN E_2 PRODUCTION IN RAW264.7 MOUSE MACROPHAGE CELLS

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Abstract

Inhibition of prostaglandin E2 (PGE_2) production in lipopolysaccharide-stimulated RAW264.7 mouse macrophage cells was assessed with an enzyme immunoassay following treatments with *Echinacea* extracts or synthesized alkylamides. Results indicated that ethanol extracts diluted in media to a concentration of 15 μg/ml from *E. angustifolia, E. pallida, E. simulata* and *E. sanguinea* significantly inhibited PGE_2 production. In further studies, PGE_2 production was significantly reduced by all synthesized alkylamides assayed at 50 μM, by Bauer alkylamides 8, 12A analog, 14, Chen alkylamide 2, and Chen alkylamide 2 analog at 25 μM and by Bauer alkylamide 14 at 10 μM. Cytotoxicity did not play a role in the noted reduction of PGE_2 production in either the *Echinacea* extracts or synthesized alkylamides. High performance liquid chromatography analysis identified individual alkylamides present at concentrations below 2.8 μM in the extracts from the six *Echinacea* species (15 μg/ml crude extract). Since active extracts contained less than 2.8 μM of specific alkylamide and our results showed that synthetic alkylamides must have a minimum concentration of 10 μM to inhibit PGE_2, it is likely that alkylamides may contribute toward the anti-inflammatory activity of *Echinacea* in a synergistic or additive manner.

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Keywords: *Echinacea purpurea*; *Echinacea angustifolia*; *Echinacea pallida*; *Echinacea tennesseensis*; *Echinacea simulata*; *Echinacea sanguinea*; Anti-inflammatory; Cytotoxicity

Introduction

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

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

The chemical diversity these plants exhibit has made it difficult for researchers to determine if *Echinacea* can be effective in treating colds and other respiratory infections (*6*). Inconsistent results have been obtained from several placebo-controlled studies designed to determine whether *Echinacea* preparations were effective in the prevention of the common cold and other upper respiratory infections. These conflicting results were perhaps due to the use of different species and plant parts, different preparations and doses, inconsistent

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times of treatment initiation, and different virus types and doses (*5*, *6*). Some studies revealed a shorter duration of cold symptoms after *Echinacea* treatment in comparison to placebo (*7*, *8*), whereas others showed no significant differences between treatment and placebo groups (*9*, *10*). Though the controversy regarding efficacy continues, studies are accumulating that indicate *Echinacea* may have anti-viral, antioxidant, and anti-inflammatory properties making it a very promising medicinal botanical (*4*, *11*, *12*).

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

Prostaglandin E2 (PGE_2) is a critical inflammatory mediator that is produced through the arachidonic acid cascade. Two cyclooxygenase isoforms, COX-1 and COX-2 catalyze the reaction converting arachidonic acid, released by phospholipase A, to PGE_2. LPS is a common endotoxin used to stimulate macrophage cells to produce PGE_2, mimicking an inflammatory response *in vitro* (*13*). The use of RAW264.7 mouse macrophage cells has been established as a reliable cell model for purposes of identifying anti-inflammatory activity (*12*).

The purpose of our study was to compare the bioactivity of species of *Echinacea* and assess levels of variability based on repeat extractions and different harvest years. It was also important to determine whether specific alkylamides play a clear role in the anti-inflammatory properties of six *Echinacea* species. To this end, we have conducted (to our knowledge) the first large scale screen of nine synthesized alkylamides found in *Echinacea*, three synthesized analogs, two of Bauer alkylamide 12 (*14*) and one of Chen alkylamide 2,

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and one synthesized isomer of Chen alkylamide1 (*12*) for their ability to reduce LPS-stimulated PGE_2 production.

Materials and Methods

Plant Material and Extraction

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

Extracts were prepared by one of two methods, either the Soxhlet method (6 hours) or room temperature shaking (24 hours). Solvents ranging in hydrophobicity were used for extraction, consisting of ethanol (70%, 95%, or 100%), water, chloroform (100%), hexane (100%), or sequential extractions. Sequential extractions were performed by extracting first with chloroform (70%, 95%, or 100%) or hexane (70%), removal of the solvent, and then evaporation. The residue plant material was re-extracted with ethanol. FNPC plant material

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was extracted with either 100% ethanol using the Soxhlet method (heating solvent to its boiling point) or shaking with 70% ethanol at room temperature. The Soxhlet method was determined to yield optimal material and was therefore used to extract NCRPIS *Echinacea* material with 95% ethanol. Upon complete drying of the extract by evaporation, the residue was re-dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at the highest concentration that was soluble. Extracts were stored at -30°C in the dark and used as stock solutions.

Endotoxin

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

Cell Culture

RAW264.7 mouse monocyte/macrophage cells were obtained from American Type Culture Collection (cat: TIB-71, Manassas, VA) and cultured as described by Hammer et al. (*15*). NIH/3T3 mouse fibroblast cells, SW480 human colon cancer cells, and HaCaT human skin cancer cells were cultured according to procedures described by Schmitt et al. (*16*).

Alkylamide Synthesis

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

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Measurement of Prostaglandin E_2

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

Cytotoxicity

Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) was used to analyze cytotoxicity following a modified version of Schmitt et al.'s protocol (*16*). RAW264.7 cells were plated into 48 well plates at a density of $0.5x10^5$cells/well and incubated at 37°C for 24 hours before treatment. Treatments were prepared by diluting the 6g of *Echinacea* extract or alkylamide in DMSO and then further

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

High Performance Liquid Chromatography Analysis

The HPLC method was described in previously published work (*17*, *23*). Briefly, into 320 µl of *Echinacea* extracts, 40 µl (1 mg/ml) *N*-isobutylundeca-2-ene-8, 10-diynamide ($C_{15}H_{21}O_2$) and 40 µl (1 mg/ml) 3, 5-dimethoxy-4-hydroxy-cinnamic acid ($C_{11}H_{12}O_5$) were added as internal standards for quantification of lipophilic metabolites and hydrophilic metabolites, respectively. Fifteen µl of each extract were injected into a HPLC. The instrumentation and solvent system for HPLC separation were the same as those previously published (23). For compound identification, Bauer alkylamides 8/9, cichoric acid, echinacoside, caftaric acid, and cynarin were purchased from Phytolab, (Vestenbergsgreuth, Germany)**;** chlorogenic acid was purchased from Sigma-Aldrich (St.Louis, MO, USA); and Bauer alkylamides 2,10-14 and ketone 20-22 were synthesized (*17*, *18*). Peaks were identified according to retention time and mass spectra obtained from LC-MS and/or GC-MS. In the absence of standards, Bauer alkylamides 1, 3, 4, 5, 7, 15, 16, 17 and ketone 24

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were identified by HPLC fractionation coupled with GC-MS analysis. Specifically, eluted HPLC fractions were collected and subsequently subjected to GC-MS analysis; six replicate runs were carried out, and appropriate peaks were pooled to ensure sufficient yield of each alkylamide. Compounds contained in each pooled fraction were identified by comparing their recorded mass spectra and online UV spectra with those from published literature (*24*). Compounds were quantified based on the internal standards. The percent repeatability and limits of detection for HPLC quantification of Bauer alkylamides 2, 8/9, 10, 11, 12, 13, 14, ketones 20, 21, 22, caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid with reference standards range between 1.64 – 2.86 % and 0.02 µg/ml - 0.16 µg/ml, respectively. The repeatability was determined by analyzing repeated injections of the standard solution (n = 6). The standard deviation values of the repeatability are less than 3%, illustrating the precision of the HPLC method.

Statistical Analysis

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

Results

Extracts from *Echinacea* Species Inhibit PGE_2 Production

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To assess anti-inflammatory properties of six *Echinacea* species, Soxhlet EtOH extracts were initially screened in RAW264.7 cells at their highest concentration (ranging in final concentrations from 21 μg/ml to 53 μg/ml) for their ability to reduce PGE_2 levels after stimulation with LPS (**Figure 1**). *Echinacea angustifolia* and *E. pallida* extracts from NCRPIS and Johnny's Selected Seeds significantly inhibited PGE_2 levels at concentrations above 21 μg/ml. Neither *E. purpurea* accession screened in **Figure 1** significantly inhibited PGE_2 (28 μg/ml of extract from NCRPIS and 24 μg/ml of extract from Johnny's Selected Seeds). Treatments analyzed without the addition of LPS reduced PGE_2 levels in *E. purpurea* and *E. angustifolia* compared to media + DMSO control. Baicalein and quercetin were included as positive controls for every PGE_2 experiment. After initial screening the extracts were diluted to 15 μg/ml in DMSO for activity comparisons across species. There was no significant difference among harvest year, accession, or Soxhlet EtOH extract preparation for *E. purpurea* (6 extracts), *E. angustifolia* (4 extracts), *E. pallida* (4 extracts), *E. sanguinea* (2 extracts), *E. simulata* (2 extracts), or *E. tennesseensis* (2 extracts), which allowed data to be pooled. Of the three medicinal species, *E. angustifolia* and *E. pallida* significantly inhibited PGE_2 levels ($p<0.05$) (**Figure 2**). Three non-medicinal species were also screened for anti-inflammatory activity. *Echinacea sanguinea* and *E. simulata* significantly reduced PGE_2 production ($p<0.001$ and $p<0.05$, respectively). Of the six species being compared in this study, *E. purpurea* and *E. tennesseensis* showed the least activity in this assay. Of the four active species that reduced PGE_2 production, none was significantly more active than the other when comparing confidence intervals across species.

Screening for Cytotoxicity of Extracts of *Echinacea* Species

To determine whether any of the NCRPIS Soxhlet EtOH extracts had the ability to arrest metabolic activity in RAW264.7 macrophage cells and to provide further evidence that the observed PGE_2 reduction with treatment of *Echinacea* extracts was not due to cytotoxicity, a parallel study using the Celltiter96 Aqueous One Solution Cell Proliferation

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Assay was conducted. **Table 1** displays an initial screening of each extract at a 1% concentration of extract diluted in media for a 24 hour incubation. This initial screening used 10-fold higher concentrations than those used in the PGE_2 screens. All extracts showed significant cytotoxicity ($p<0.0001$), with 25% - 72% survival at the 1% concentration (ranging from 240 to 1102 μg/ml of extract) and 24 hour incubation.

All extracts cytotoxic at the 1% concentration were screened again at the 0.1% concentration of extract diluted in media (concentrations ranging from 24 to 110 μg/ml of extract) after a 24 hour incubation. Results in **Table 1** show that only one extract of *E. pallida* from the 2003 harvest (PI631293) remained significantly cytotoxic at the 0.1% dilution after a 24 hour incubation, but when this same extract was screened for cytotoxicity at the same dilution after an 8 hour incubation, equivalent to the incubation period in the PGE_2 assay, no significant cytotoxicity remained ($p = 0.9968$). The screens conducted at 0.1% concentration matched concentrations used in PGE_2 screens. 70%, 95% or 100%),

Cytotoxicity Screen of Frontier National Product Co-op *Echinacea purpurea* Extracts

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

Synthesized Alkylamides from *Echinacea* Species Inhibit PGE_2 Production

To further probe the inhibition of PGE_2 production in RAW264.7 cells observed with treatments of *Echinacea* extracts, another PGE_2 screening was conducted on chemically

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synthesized alkylamides, one of *Echinacea*'s major classes of bioactive constituents. Data from **Table 2** shows that all of the synthesized alkylamides screened significantly inhibited the production of PGE_2 ($p<0.001$) at 50 μM. At 25 μM, Bauer alkylamide 8, Bauer alkylamide 12A analog, Bauer alkylamide 14, Chen alkylamide 2, and the Chen alkylamide 2 analog significantly reduced PGE_2 levels ($p<0.05$). Only Bauer alkylamide 14 significantly inhibited PGE_2 production at 10 μM ($p<0.05$). A subsequent PGE_2 EIA experiment was conducted to attempt to determine if PGE_2 could inhibit at a concentrations less that 10 μM. After having stored Bauer alkylamide 14 at -24°C for one year, HPLC analysis indicated Bauer alkylamide 14 had degraded to 31% of the original concentration. The concentrations screened for PGE_2 production were 3.1 μM and 0.230 μM, and Bauer alkylamide 14 was unable to significantly inhibit PGE_2 at these concentrations (data not shown).

Screening for Cytotoxicity of Synthesized Alkylamides

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

HPLC Analysis of *Echinacea* Extracts at 15 μg/ml

High performance liquid chromatography was performed with 15 μg/ml of *Echinacea* extracts from NCRPIS to identify and analyze innate concentrations of known constituents (**Table 3**). *Echinacea angustifolia*, *E. sanguinea*, *E. purpurea*, and *E. tennesseensis* contained greater quantities of Bauer alkylamides than of ketones or caffeic-acid derivatives. Bauer alkylamides 3 and 8-13 were present in *E. angustifolia* at concentrations greater than 0.1 μM, with Bauer alkylamide 9 reaching concentrations of 2.8 μM and 1.6 μM in the two extracts analyzed. All accessions of *E. sanguinea* also contained Bauer alkylamides 3, 8, 9, 12, 13, 14, and 17 at concentrations ranging from 0.16 to 1.15 μM.

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Two different accessions of *E. purpurea* (PI 63665 and PI 631307) were analyzed by HPLC and were found to both contain similar alkylamides, including Bauer alkylamides 1-3 and 7-9 (>0.1 μM). Cichoric acid was present in one extract of each accession from the 2003 harvest of *E. purpurea*. *Echinacea tennesseensis* (2003 and 2004 harvest) contained Bauer alkylamide 12 at concentrations greater than 0.3 μM and Bauer alkylamide 13 with concentrations greater than 0.6 μM. Two species, *E. pallida* and *E. simulata*, were determined to possess greater amounts of ketones than of alkylamides. *Echinacea pallida* had concentrations exceeding 0.1 μM for ketones 22 and 24. Ketone 22 was also present in *E. simulata* extracts (>0.1 μM of ketone). Chen alkylamide 1, Chen alkylamide 1 analog, Chen alkylamide 2 analog, Bauer alkylamide 12A analog, and Bauer alkylamide 12B analog from Table 2 were not detected in this analysis, but may be present in concentrations below the detection limits of our instrument. Some alkylamides, ketones, and caffeic acid derivatives were present in only certain extracts within the species and are identified in Table 3.

Discussion

The results presented in this study demonstrate the inhibition of PGE_2 by several *Echinacea* species, which may be one process contributing to the reported anti-inflammatory capabilities of these herbs (*12*). All Soxhlet EtOH extracts from the six species screened, when tested at their highest concentration, reduced PGE_2 levels, except *E. purpurea*. To compare inhibition potential among these species, extracts were diluted to 15 μg/ml in DMSO, with *E. angustifolia*, *E. pallida*, *E. sanguinea*, and *E. simulata* significantly inhibiting the production of PGE_2 in LPS induced RAW264.7 mouse macrophage cells. *Echinacea purpurea* extracts (15 μg/ml) did not significantly inhibit PGE_2, which was notable since many *Echinacea* supplements contain *E. purpurea* as a major ingredient (*21*). Another interesting finding was that variability between species was greater than that observed between repeat extractions or harvest years. Results obtained from screening

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Echinacea extracts in RAW264.7 cells for cytotoxicity coincide with observations from NIH/3T3 mouse fibroblast cells, SW480 human colon cancer cells, and HaCaT human skin cancer cells, which found these extracts to be cytotoxic only at high concentrations (>240 μg/ml of extract).

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

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

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The data presented strengthen the research indicating that alkylamides present in *Echinacea* contribute to immunomodulatory properties dealing with regulation of inflammation (*27*).

Questions as to how alkylamides act together and in concert with other constituents arise from our HPLC analysis. Our data identify alkylamides present in crude *Echinacea* extracts at concentrations below 2.8 μM, which is well under the 10 μM concentration observed for inhibitory activity of PGE_2 seen with chemically synthesized Bauer alkylamide 14. Regardless, several of the crude extracts of *Echinacea*, containing a variety of alkylamides, ketones, and caffeic-acid derivatives at low concentrations, were able to significantly inhibit PGE_2 production. For example, *E. sanguinea* extracts at 15 μg/ml, containing Bauer alkylamides 8, 12, 13, and 14, at higher concentrations than other constituents present, showed the greatest inhibition of PGE_2 among the species we evaluated, with a percent reduction of 59%, whereas none of these alkylamides would be able to inhibit PGE_2 production alone at such low concentrations. Therefore, we hypothesize that the noted inhibition of PGE_2 and, by extension, the anti-inflammatory properties found in *Echinacea* extracts was not simply due to one constituent, but several acting in a synergistic or additive manner. Synergism has been reported previously between alkylamides and caffeic acid derivatives and their ability to inhibit the oxidation of low-density lipoproteins, as an indicator of antioxidant activity (*4*). Our HPLC analysis revealed no clear pattern of identifiable constituents that led to the observed inhibition of PGE_2 by extracts from *E. angustifolia*, *E. pallida*, *E. simulata*, or *E. sanguinea*, indicating that more research is needed to understand the complex nature of interacting constituents within each species and to determine mechanisms behind the identified PGE_2 inhibition. A possible explanation for the resemblance of constituents found in *E. pallida* and *E. simulata*, may be related to the hypothesis that *E. simulata* is one of the diploid progenitors of the tetraploid species, *E. pallida* (*30*). A PGE_2 screening of synthesized ketones and caffeic-acid derivatives

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individually may lead to a better understanding of *Echinacea's* anti-inflammatory capabilities.

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

Studies are providing convincing evidence that alkylamides can play important roles in the bioactivity observed in *Echinacea* species, and questions about the bioavailability of this class of constituents are still being answered. Alkylamides have been shown to be readily bioavailable through the Caco-2 cell monolayer, more so than other active compounds, such as the caffeic-acid derivatives found in *Echinacea* (*31*). Another study had

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previously supported these data by finding that (2E, 4E, 8Z, 10Z)-N-isobutyldodeca -2, 4, 8, 10-tetraenamide could be completely transported from the apical to the basolateral side of the Caco-2 monolayer, with no significant metabolism occurring (*32*). Results from a study investigating the metabolism of natural and synthetic alkylamides from *Echinacea* by using human liver microsomes determined that cytochrome P450 mediated epoxidation, hydroxylation, and dealkylation of alkylamides occurred (*33*). A recent human study analyzed 11 human subjects for bioavailability of an oral administration of a 60% ethanolic extract of *E. angustifolia* root, which was known to contain six identified alkylamides, showing that after 30 minutes one of the six alkylamides, dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide, appeared in plasma samples at 10.88 ng/ml for a 2.5 ml dose (*34*). Another human study analyzed 9 volunteers who consumed tablets of 675mg of *E. purpurea* and 600 mg of *E. angustifolia*, after a high fat breakfast or after a fast, for alkylamide content in their plasma (*35*). Total 2, 4-diene alkylamides were found in the plasma from the high fat group with a maximum concentration ranging from 60 - 1137 ng/ml. Although it is becoming increasingly evident that alkylamides are bioavailable, more experimentation is warranted to determine whether alkylamides can exert anti-inflammatory or other immunomodulatory effects at the low concentration of 10 μM, which was the lowest concentration found to be bioactive in our PGE_2 studies. Without more definitive bioavailability data, a difficulty arises in making assumptions about alkylamides anti-inflammatory properties based on cell culture data.

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

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alkylamides, it is clear that the presence of individual alkylamides alone cannot explain the observed anti-inflammatory activity.

Abbreviations Used

E. = *Echinacea*

PI = Plant Introduction

FNPC = Frontier Natural Products Co-op

NCRPIS = North Central Regional Plant Introduction Station

PGE_2 = Prostaglandin E2

LPS = Lipopolysaccharide

Safety

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

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Note

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Table 1. Cytotoxicity screening of Soxhlet EtOH extracts from six *Echinacea* species

species	harvest year	accession	1% concn (µg/ml)	% survival (95% CI), p value	0.1% concn (µg/ml)	% survival (95% CI), p value
E. angustifolia	2005	JS	535	71 (53, 97), **0.0166**	54	92 (74, 116), 0.9792
E. purpurea	2003	PI633665	518	65 (48, 88), **0.0004**	52	104 (79, 139), 1.0000
E. purpurea	2005	JS	240	72 (53, 97), **0.0181**	24	105 (78, 139), 1.0000
E. pallida	2003	PI631293	579	68 (50, 92), **0.0021**	58	75 (56, 99), **0.0365**
E. pallida	2005	JS	359	32 (26, 40), **<0.0001**	36	133 (100, 177), 0.0457
E. tennesseensis	2003	PI631250	950	66 (53, 83), **<0.0001**	95	112 (84,149), 0.9431
E. simulata	2003	PI631251	1101	25 (20, 32), **<0.0001**	110	102 (77, 136), 1.0000
E. simulata	2004	PI631251	1102	25 (20, 32), **<0.0001**	110	110 (83, 146), 0.9817
E. sanguinea	2003	PI633672	834	36 (28, 45), **<0.0001**	83	93 (74, 116), 0.9874
E. sanguinea	2004	PI633672	672	34 (27, 43), **<0.0001**	67	96 (73, 128), 1.0000

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

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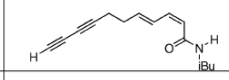
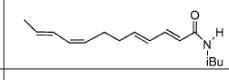
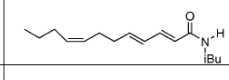
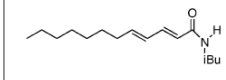
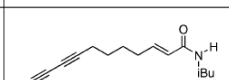
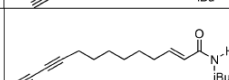
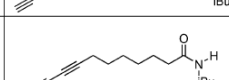
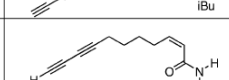
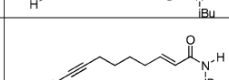
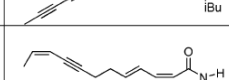
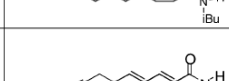
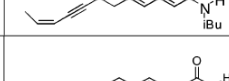
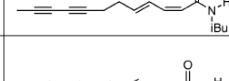
Table 2. Inhibition of PGE_2 production by alkylamide

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

* p-value for comparison of alkylamide treatment to control (media + DMSO + LPS). Bolded p-value represent statistical significance as defined as a p-value <0.05. Media + DMSO + LPS control set to 0% reduction. There was no difference between extracts in media alone having an overall % reduction (95% CI) of 2 (-27, 24) and media + DMSO controls. Baicalein (6 µM) and Quercetin (10 µM) were used as positive controls having overall % reductions (95% CI) of 72 (62, 79) and 88 (85, 91), respectively. All samples in the table are treated with 1 µg/ml LPS. Alkylamide treatments did not affect PGE2 levels without LPS treatment (Data not shown). Bauer alkylamides are found in Bauer et al. (14) and **Chen alkylamides from Chen et al. (12). Isomers and analogs of naturally occurring alkylamides are indicated with the + symbol and have not been detected to date from *Echinacea* species extracts in our laboratories.

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Table 3. HPLC analysis of Soxhlet EtOH extracts of *Echinacea* species (15 µg/ml)

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

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

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Figure Legends

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

Figure 2. Inhibition of PGE_2 production by extracts of three medicinal and three non-medicinal species of *Echinacea* (diluted to 15 μg/ml of extract in DMSO) obtained from NCRPIS with different harvest dates and accession numbers. Each species mean was represented by a bar (2-6 replicates each) and variability was represented as 95% confidence intervals of the mean. Treatments analyzed without the addition of LPS did not affect PGE_2 levels with values for PGE_2 as % of media + DMSO control less than 20% (data not shown). One *E. angustifolia* extract, from the 2004 harvest, treated without LPS, was excluded from the analysis based on the optical density data point being outside the standard curve. * $p<0.05$ and ** $p<0.001$ for comparison of extract to control (media + DMSO + LPS).

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Figure 1. Inhibition of PGE_2 production by medicinal species Soxhlet EtOH extracts

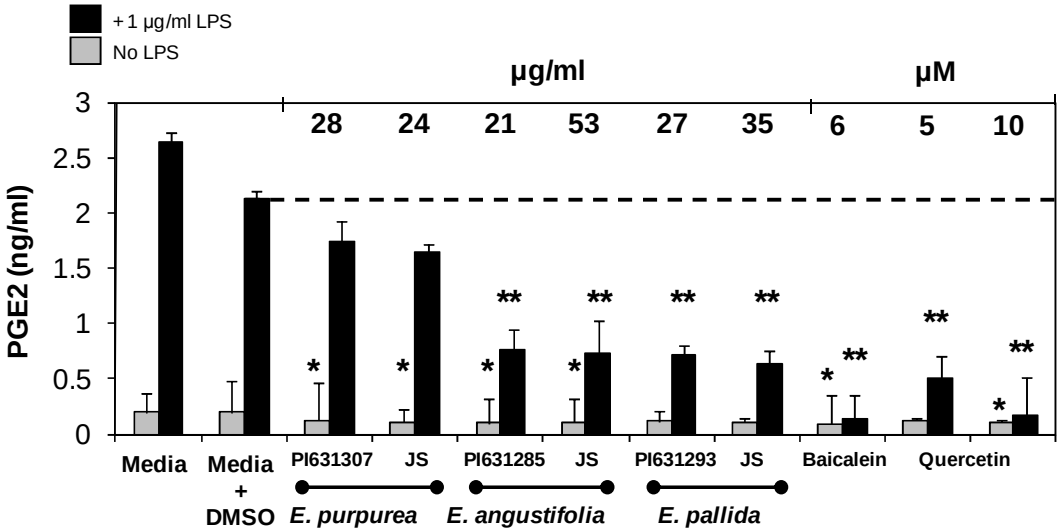

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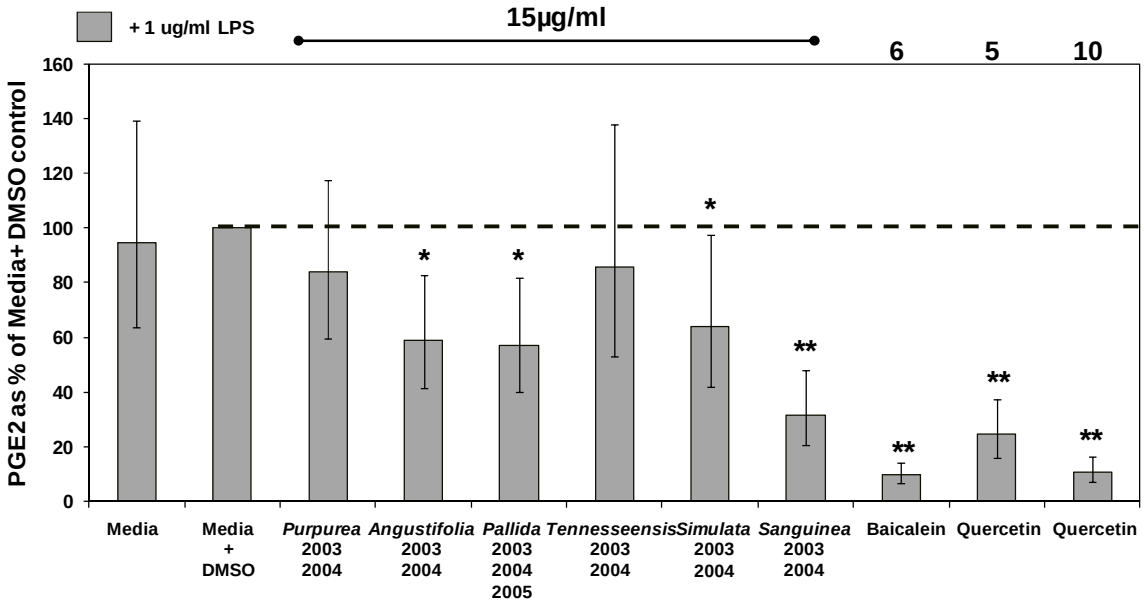
Figure 2. Comparison of PGE_2 inhibition among *Echinacea* species extracts



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CHAPTER 4: ENDOGENOUS LEVELS OF *ECHINACEA* ALKYLAMIDES AND KETONES ARE IMPORTANT CONTRIBUTORS TO THE INHIBITION OF PROSTAGLANDIN E2 AND NITRIC OXIDE PRODUCITON 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 (PGE_2) 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 PGE_2 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 PGE_2 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 PGE_2 inhibition. Chemically synthesized Bauer ketones 21 and 23 at 1 μM each significantly inhibited both PGE_2 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

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Bauer alkylamide 11 making up 96% of third round fraction 3E40. Synthetic Bauer ketone 23 inhibited PGE_2 production to 83 % of control and synthetic Bauer alkylamide 11 significantly inhibited PGE_2 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.

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 PGE_2, 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

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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 PGE_2 production when macrophage cells are stimulated by lipopolysaccharide (LPS) makes this eicosanoid 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 PGE_2 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 PGE_2 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 PGE_2 assay with *E. angustifolia*.

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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 though the semi-preparative HPLC fractionation. The extracts were stored at -20ºC overnight prior to fractionation.

Semi-Preparative HPLC Fractionation

The 95 % ethanol extracts of *Echinacea* were filtered through a 0.45 µm 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

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Coulter, Fullerton, CA) using a YMC-PACK ODS-AM 250*10 mm I.D. s-5 μm, 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.

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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 split-less 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 x 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

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by Hammer et al. (20) with the exception of maintaining optimal growth conditions at 95% humidity.

Measurement of Prostaglandin E_2

Prostaglandin E_2 production was analyzed using PGE_2 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. tennesseensis* 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*

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species analyzed. Each fraction was screened for cytotoxicity at concentrations comparable to those used in the PGE_2 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 50μM, 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 PGE_2 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 ± standard error, normalizing the (Media + DMSO + LPS) control to 100% PGE_2 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 ± 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 PGE_2 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 PGE_2 production in RAW264.7 mouse macrophage cells. The analysis yielded one fraction, Fraction 3, capable of significantly inhibiting PGE_2 production (36.8 % of control) at a concentration of 5 μg/ml (**Figure 1**). Another interesting observation was that Fraction 3, at a concentration of 5 μg/ml, inhibited PGE_2 production to a

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greater extent than the initial ethanol extract of *E. pallida* at a concentration of 25 μg/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 μg/ml to 1 μg/ml, inhibition of PGE_2 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 PGE_2 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 PGE_2 production (**Figure 2B**). This analysis indicated that Bauer ketones 23 and 24 were the most important of the identified ketones in partially explaining the PGE_2 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 PGE_2 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 PGE_2 identified with fraction 3. It was determined that Bauer ketones 21 and 23 were capable of significant inhibition of PGE_2 ($p<0.05$) 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 PGE_2 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 PGE_2 inhibitory capabilities. Fraction 3, containing Bauer alkylamides 12, 13, 14, 16, and 17, was able to

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significantly inhibit PGE_2 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 PGE_2 production further dilutions were not carried out.

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

Inhibition of PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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**). PGE_2 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 PGE_2 production observed with fraction 3E. The combination of Bauer alkylamides 10 and 11 at the

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concentrations detected by the HPLC analysis of fraction 3E, inhibited PGE_2 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 PGE_2 production assay were significantly inhibitory at the concentrations found in fraction 3E, but additive effects were not detected in the PGE_2 production screen. The combination of alkylamides found in fraction 3D was not carried out as fraction 3E was more effective at inhibiting PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 production at a concentration of 1 μg/ml, fraction 3E33 that inhibited PGE_2 production at 14.1 % of control, and fraction 3E40 that inhibited PGE_2 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 PGE_2 production assay (**Table 3**). Fractions 3D32, 3E33 and 3E40 were selected for GC-MS analysis due to their ability to significantly inhibit PGE_2 production at a concentration of 1 μg/ml. Two fractions, fraction 3D28 and 3E38, were

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also selected for further analysis because they were fractions that did not significantly inhibit PGE_2 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 PGE_2 and NO production at the concentrations present in their respective third round fractions. Synthetic Bauer ketone 23 was shown to significantly inhibit PGE_2 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 PGE_2 production remained significant whereas NO production was not significantly inhibited (55.2 % of control and 68.2% of control, respectively) (**Figure 9 for PGE_2 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 PGE_2 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 PGE_2 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,

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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 PGE_2 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 PGE_2 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 PGE_2 production at concentrations present in their respective *E. angustifolia* fractions, are capable of inhibiting and partially explaining the significant PGE_2 suppression identified with their respective plant derived fractions. Also, Bauer ketones 23 and 24 were identified as key components responsible for the inhibition of PGE_2 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 PGE_2 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 PGE_2 production to a significant degree at concentrations below 5 μg/ml. Therefore, studies on these two species ceased after the first round of fractionation.

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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 PGE_2 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 PGE_2 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 PGE_2 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 IC_{50} 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 32×10^{-6} cms^{-1} and 10×10^{-6} 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

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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 PGE_2 production, as well as inhibit the degradation of IkB and further DNA binding of NF-kB (*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 PGE_2 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 PGE_2 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 PGE_2 inhibitory capabilities. Synthetic Bauer alkylamide 10 and 11 individually were capable of significant inhibition of PGE_2 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 PGE_2 inhibitory capabilities. Previous studies in our lab found that individually, chemically synthesized Bauer alkylamide 10 and 11 were not capable of significant PGE_2 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

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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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 ID_{50} of

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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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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

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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.

Abbreviations Used

E. = *Echinacea*

PI = Plant Introduction

DMSO = Dimethyl Sulfoxide

PGE_2 = Prostaglandin E2

NO = Nitric Oxide

LPS = Lipopolysaccharide

GC-MS = Gas Chromatography Mass Spectrometry

HPLC = High Performance Liquid Chromatography

ID_{50} = Inhibition Dose producing 50 % inhibition

Safety

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

Acknowledgment

The authors would like to thank all members of the Center for Research on Botanical Dietary Supplements at Iowa State University and the University of Iowa for their

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cooperation and ongoing advice in directing the progress of this research. A special thanks for the gift of *Echinacea* plant material from the North Central Regional Plant Introduction Station at Iowa State University (Ames, IA). The authors would also like to acknowledge George Kraus and Jaehoon Bae for providing the chemically synthesized alkylamides and ketones for this study.

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Table 1. Inhibition of LPS induced PGE_2 production and cytotoxicity analysis after treatments with *E. purpurea* and *E. tennesseensis* fractions[a]

	Anti-inflammatory (PGE_2)			Cytotoxicity		
Species Fraction Accession	**Concentration (µg/ml)**	**% of Control ± SE**	**p-value**	**Concentration (µg/ml)**	**% Control ± SE**	**p-value**
E. purpurea **Fraction 1** PI631307	274	122. ± 22	0.62	274	101 ± 2	0.81
E. purpurea **Fraction 2** PI631307	89	134 ± 10	0.38	89	98 ± 3	0.49
E. purpurea **Fraction 3** PI631307	75	65 ± 21	0.08	75	105 ± 2	0.23
E. purpurea **Fraction 4** PI631307	41	112 ± 11	0.75	41	98 ± 4	0.56
E. purpurea **Fraction 5** PI631307	95	118 ± 21	0.68	95	96 ± 2	0.33
E. purpurea **Fraction 6** PI631307	96	123 ± 15	0.56	96	101 ± 4	0.87
E. purpurea **Fraction 7** PI631307	23	137 ± 39	0.53	23	94 ± 1	0.14
E. purpurea **Ethanol Extract** PI631307	25	48 ± 13	**0.02**	25	105 ± 4	1.0
E. tennesseensis **Fraction 1** PI631250	271	87 ± 9	0.48	577	94 ± 3	0.11
E. tennesseensis **Fraction 2** PI631250	0.3	109 ± 11	0.72	0.3	96 ± 5	0.30
E. tennesseensis **Fraction 2** PI631250	0.14	104 ± 13	0.91			
E. tennesseensis **Fraction 3** PI631250	41	6 ± 2	**<0.0001**	41	96 ± 3	0.77
E. tennesseensis **Fraction 3** PI631250	20	24 ± 10	**<0.0001**			
E. tennesseensis **Fraction 3** PI631250	5	90 ± 21	0.48			
E. tennesseensis **Fraction 4** PI631250	8	119 ± 4	0.43	17	97 ± 3	0.38
E. tennesseensis **Fraction 5** PI631250	11	128 ± 14	0.28	23	89 ± 3	**0.01**
E. tennesseensis **Ethanol Extract** PI631250	25	89 ± 9	0.56	59	89 ± 1	**0.01**

[a] HPLC was used to fractionate a 2005 extract of *E. purpurea* (PI631370) and a 2005 extract of *E. tennesseensis* (PI631250), yielding no significant inhibition of PGE_2 production at concentrations lower than 20 µg/ml. All treatments were compared to media + DMSO control that was set at 100% for both PGE_2 analysis and cytotoxicity. For *E. purpurea* and E. tennesseensis, 100% of control for PGE_2 was 1.7 ng/ml and 2.8 ng/ml, respectively. Baicalein (6 µM) was the positive control for the PGE_2 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 PGE_2 and cytotoxicity analyses. Cytotoxicity was not performed on dilutions of fractions when the higher concentrations were not cytotoxic.

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

	Fraction 3D		Fraction 3E	
	Metabolite Concentration		Metabolite Concentration	
Metabolite	**(µg/ml)**	**(µM)**	**(µg/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

[a]HPLC analysis identified alkylamides present in 10 µg/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*).

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

Echinacea angustifolia 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

[a]GC-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.

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Table 4. Change in PGE_2 activity over storage time with third round *E. angustifolia* fractions

E. angustifolia Fraction	PGE_2 production (% of control) August 2007	PGE_2 production (% of control) June 2008
3E33	$14.1 \pm 6.9^{**}$	133.1 ± 43.5
3E40	$18.4 \pm 6.1^{**}$	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 PGE_2 analysis in 2007 and again after PGE_2 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.

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Figure Legends

Figure 1. Fraction 3 from a 2005 extract of *Echinacea pallida* (PI631293) significantly inhibited PGE_2 production in RAW264.7 cells. The black bars represent PGE_2 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 PGE_2 production ($p \geq 0.21$). The treatments without LPS were compared to the media + DMSO control set at 100 % (0.2 ng/ml). Media alone did not inhibit PGE_2 production (104 % of control). Baicalein and quercetin were used as positive controls and showed significant inhibition of PGE_2 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 PGE_2 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 PGE_2 levels after induction with 1 μg/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 PGE_2 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 PGE_2 production (99 % of control). * and ** are representative of $p<0.05$ and $p<0.001$. Each bar represents % of control ± standard error. Quercetin was used as the positive control.

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Figure 3. (**A**) Structures and nomenclature for Bauer ketones identified in Fraction 3 from *E. pallida*. (**B**) Significant inhibition of LPS induced PGE_2 and NO production in RAW264.7 cells after treatment with chemically synthesized Bauer ketones 21 and 23. The black bars represent PGE_2 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 % PGE_2 production (3.7 ng/ml) and NO production (12.4 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE_2 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 % PGE_2 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 PGE_2 and NO production ($p<0.0001$). * 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 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 PGE_2 production. Numbers in parenthesis indicate % of control ± standard error of PGE_2 production compared to the media + DMSO + LPS control set at 100% PGE_2 production. * and ** are representative of $p<0.05$ and $p<0.001$. See figures 4-8 for details on PGE_2 data including concentrations studied.

Figure 5. Inhibition of LPS induced PGE_2 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 PGE_2 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 % PGE_2 production (2.2

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ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE_2 with fractions 1, 3, 4, and ethanol extract ($p \leq 0.035$). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 production (0.1 ng/ml). Grey bars symbolize cell survival compared to the media + DMSO control set at 100 % cell survival. Baicalein was used as the positive control in the PGE_2 analysis and showed significant inhibition of PGE_2 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 PGE_2 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 PGE_2 production in RAW264.7 cells after treatments with second round fractions from fraction 3 of *E. angustifolia* (from figure 4). The bars represent PGE_2 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 % PGE_2 production (1.8 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE_2 with fraction 3 and 3E ($p \leq 0.029$). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 production (0.1 ng/ml). Baicalein and quercetin were used as positive controls. Parallel cytoxicity 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

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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 PGE_2 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 % PGE_2 production (4.4 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE_2 with fraction 3E ($p = 0.0275$). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 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 PGE_2 production analysis after treatments with *E. angustifolia* third round D fractions. The black bars represent PGE_2 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 % PGE_2 production (2.8 ng/ml). Treatments were also performed without LPS induction showing significant reduction of PGE_2 with fractions 3D, 3D26, 3D27, 3D30, and 3D31 ($p \leq 0.04$). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 production (0.2 ng/ml). Baicalein was used as a positive control ($p<0.05$). Parallel cytoxicity 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 PGE_2 production after treatments with *E. angustifolia* third round E fractions. The black bars represent PGE_2 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 % PGE_2 production (1.9 ng/ml). Treatments were also performed without LPS induction

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showing significant reduction of PGE_2 with fractions 3E, 3E33, 3E34, 3E36, 3E37, and 3E38 ($p \leq 0.027$). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 production (0.1 ng/ml). Baicalein was used as a positive control ($p<0.05$). Parallel cytoxicity 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 PGE_2 production after treatment of synthetic Bauer ketone 23 at concentration present in third round *E. angustifolia* fraction 3D32. The black bars represent PGE_2 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 % PGE_2 production (3.8 ng/ml). Treatments were also performed without LPS induction showing no significant differences in PGE_2 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 PGE_2 and NO production in RAW264.7 cells after treatment with chemically synthesized Bauer alkylamide 11. The black bars represent PGE_2 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 % PGE_2 production (4.7 ng/ml) and NO production (11.6 ng/ml). Treatments were also performed without LPS induction showing no significant differences in PGE_2 or NO production. ND indicates analysis not determined. Quercetin was used as a positive control for both studies and showed significant inhibition of PGE_2 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

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yielding no significant cytotoxicity with Bauer alkylamide 11 at the concentrations screened (data not shown).

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Figure 1.

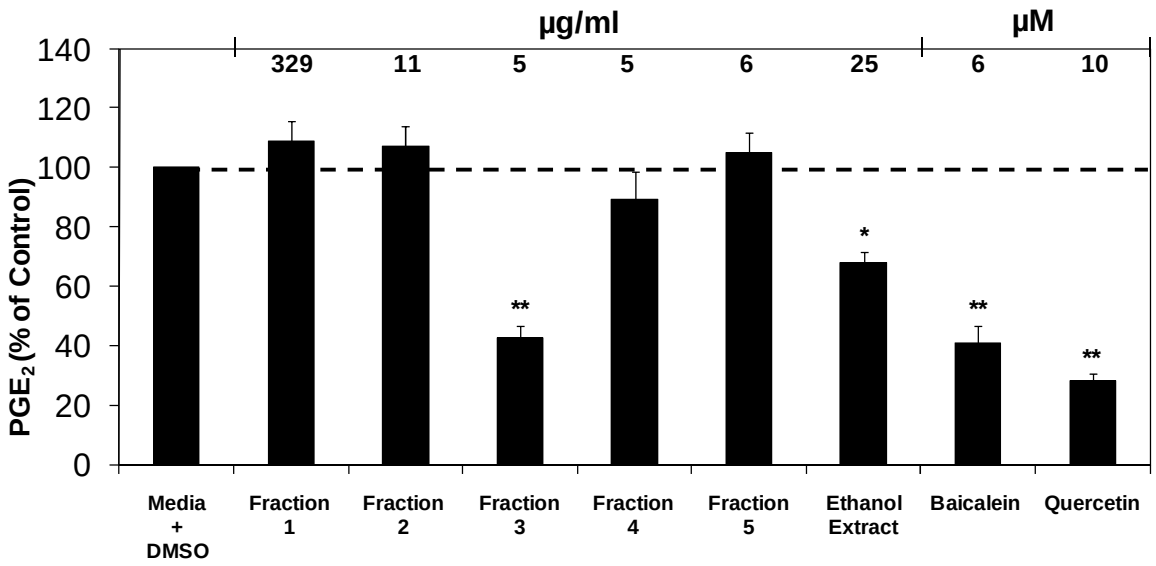

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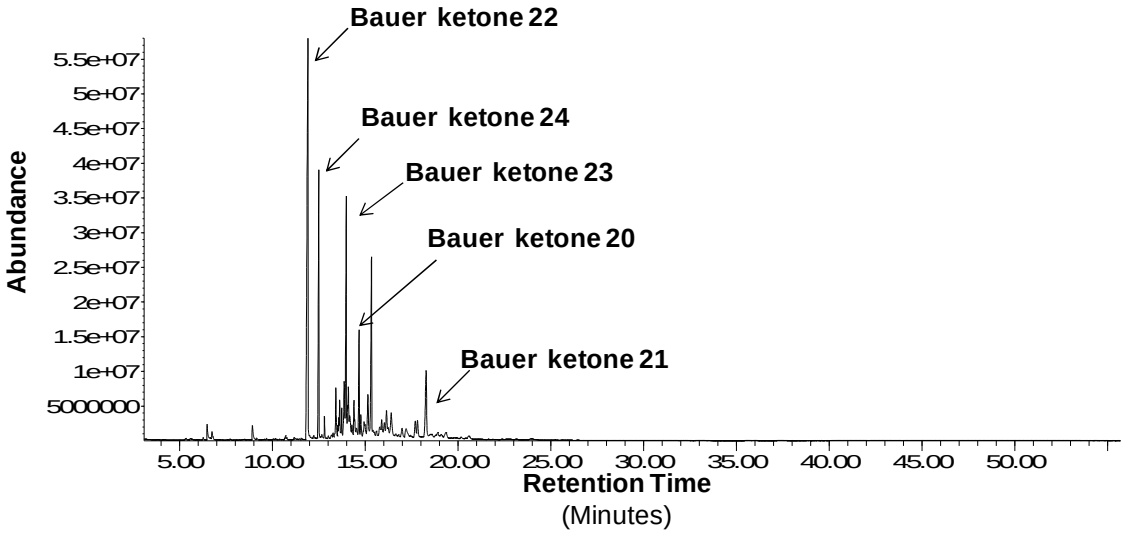
Figure 2.

A



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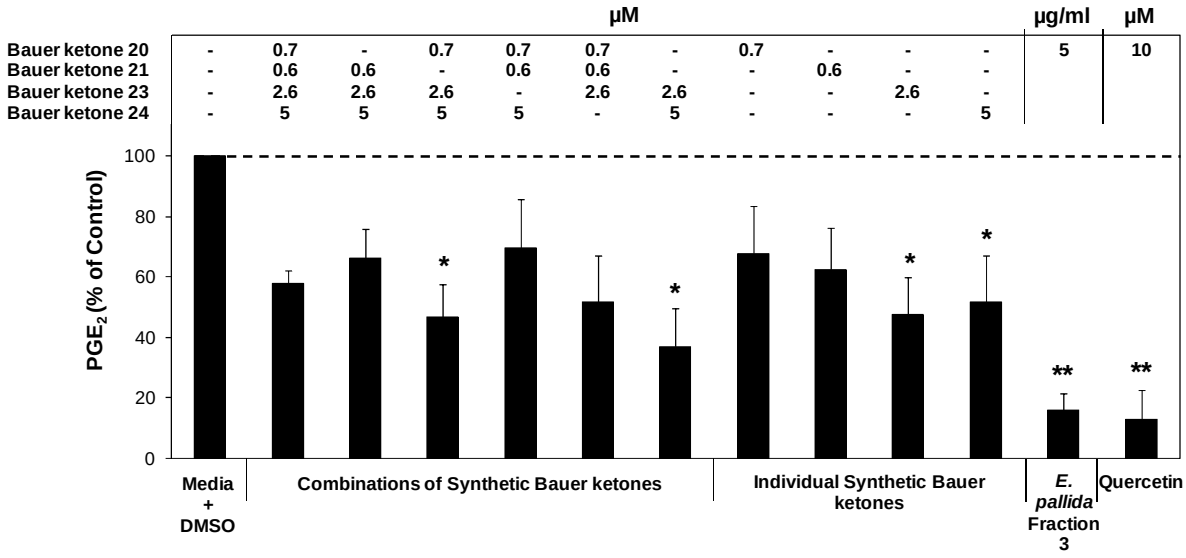

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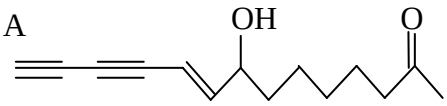
Figure 3.

A



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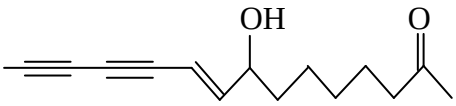
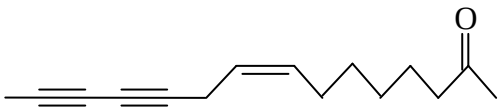
(Bauer ketone 20)



8-hydroxypentadeca-9E-ene-11,13-diyn-2-one
(Bauer ketone 21)



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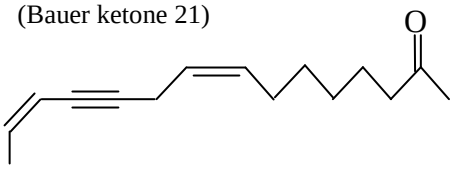
(Bauer ketone 23)



Pentadeca-8Z-ene-11,13-diyn-2-one
(Bauer ketone 24)

B

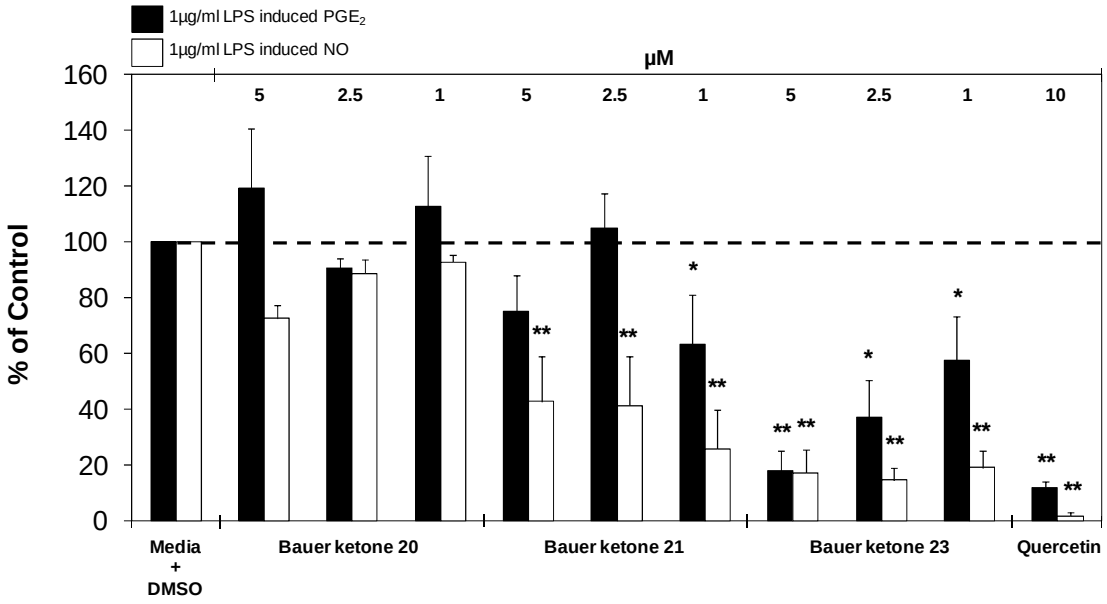


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Figure 4.

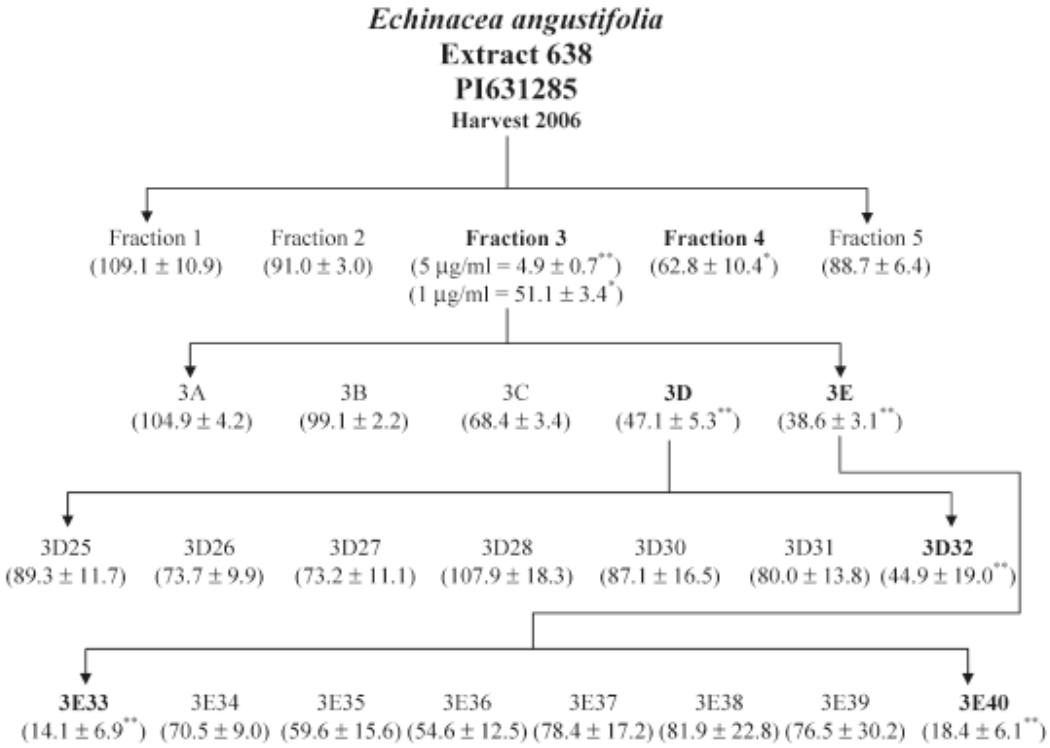

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Figure 5.

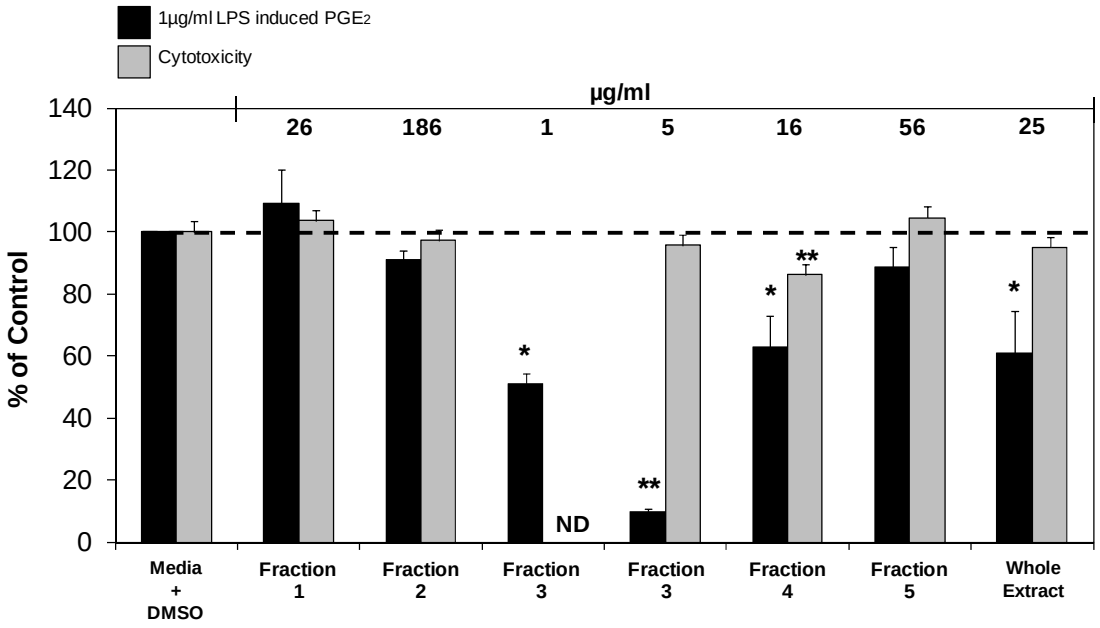

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Figure 6.

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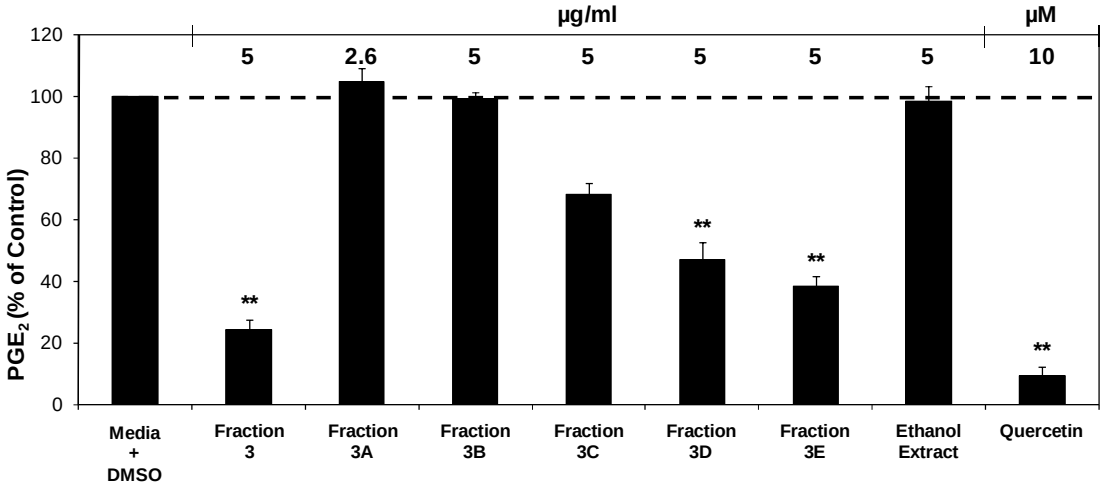

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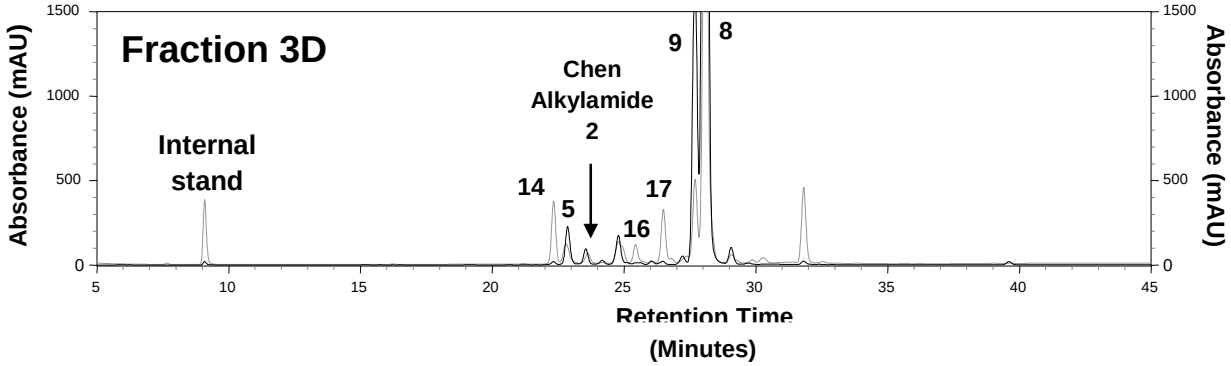

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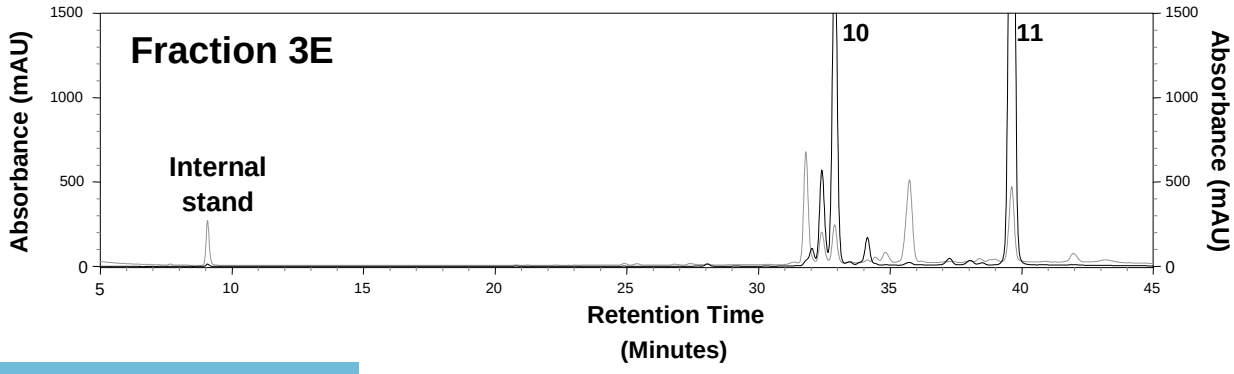

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Figure 7.

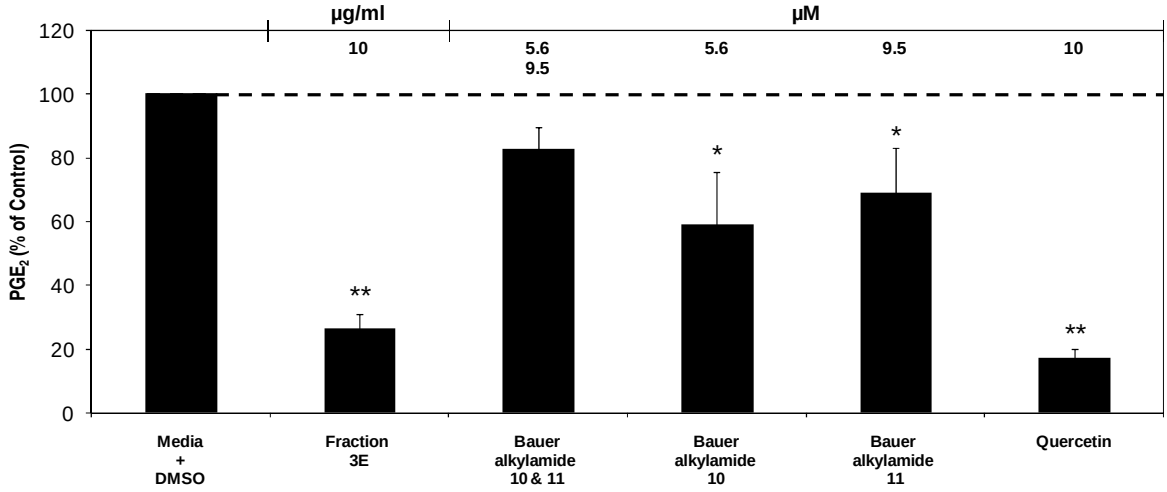

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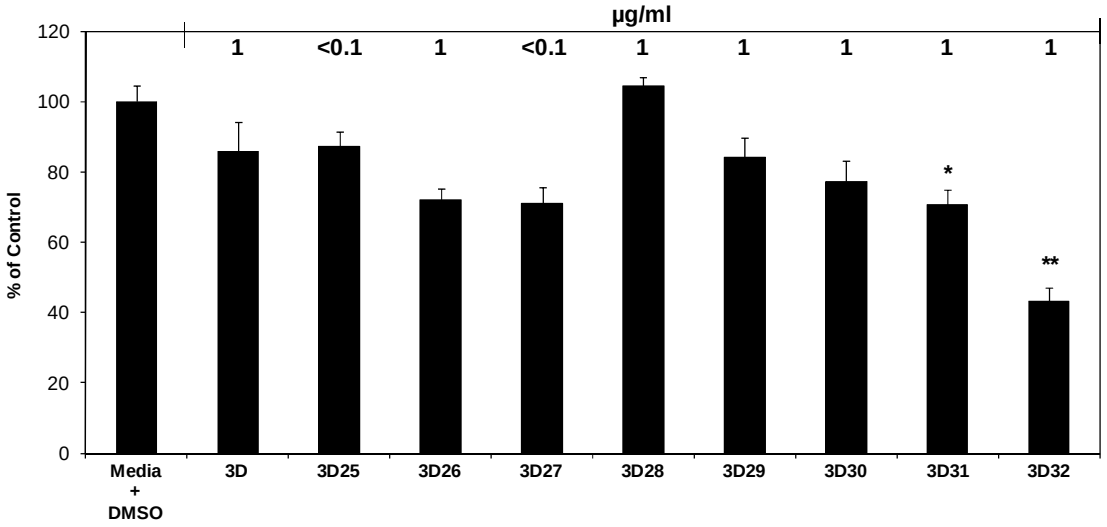
Figure 8.

A



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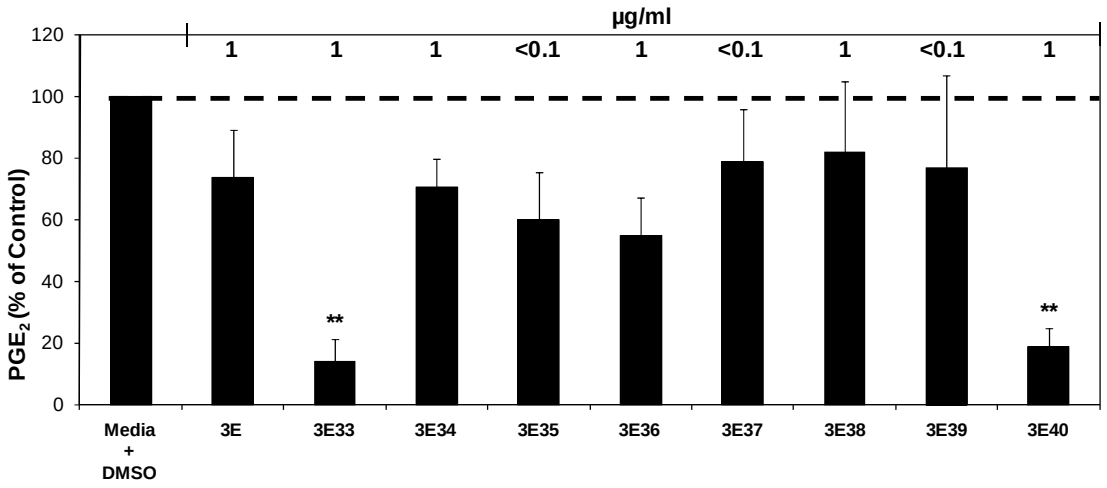

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Figure 9.

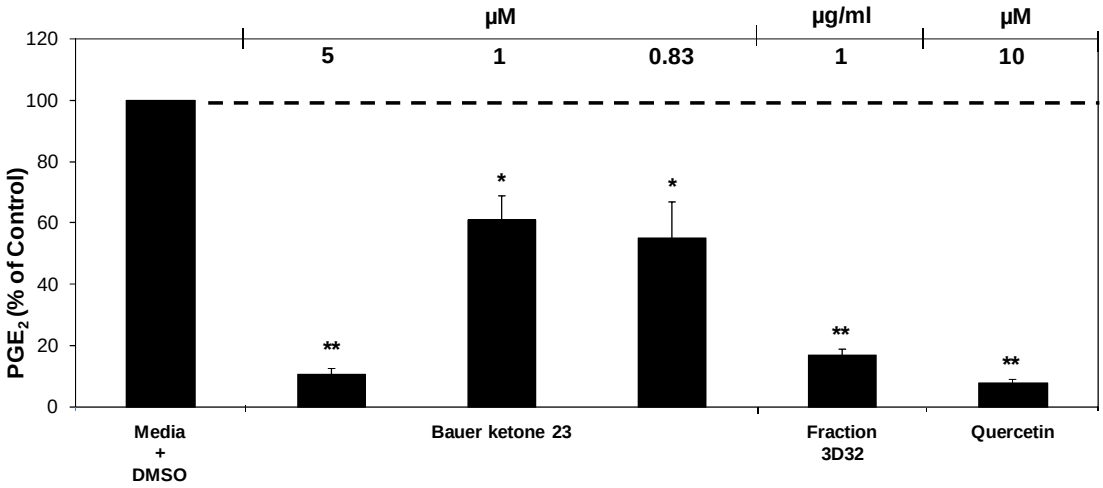

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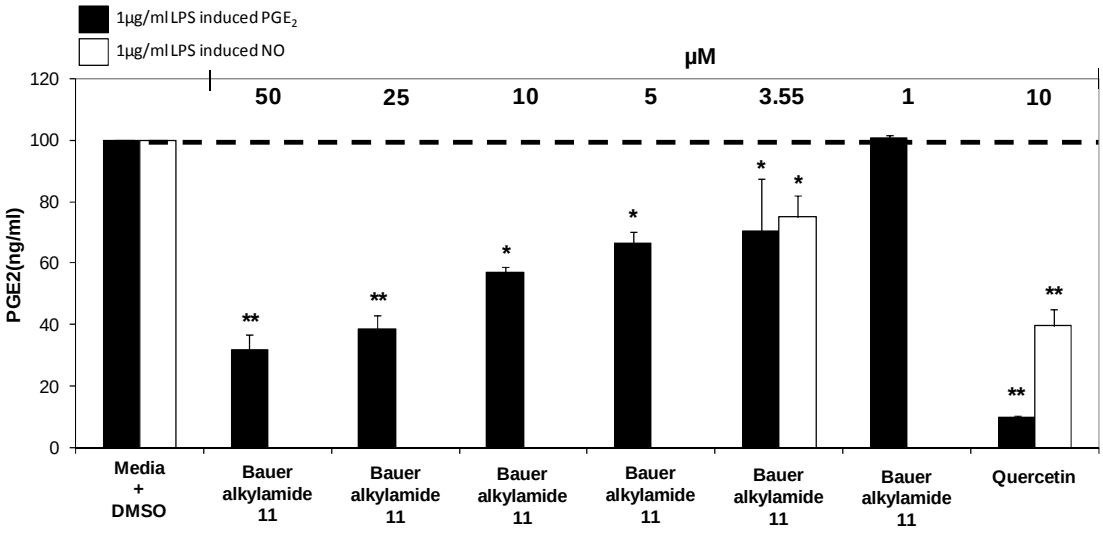
Figure 10.



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CHAPTER 5: ENRICHMENT OF *ECHINACEA ANGUSTIFOLIA* WITH BAUER ALKYLAMIDE 11 AND BAUER KETONE 23 INCREASED ANTI-INFLAMMATORY POTENTIAL THROUGH INTERFERING WITH COX-2 ACTIVITY

Modified from a paper to be submitted to the *Journal of Agricultural and Food Chemistry*

Carlie A. LaLone, Nan Huang, Ludmila Rizshsky, Man-Yu Yum, Navarozedeep Singh, Cathy Hauck, Basil J. Nikolau, Eve S. Wurtele, Marian L. Kohut, Patricia A. Murphy, and Diane F. Birt

Abstract

Previous studies have indicated that Bauer alkylamide 11 and Bauer ketone 23 were key compounds from *Echinacea angustifolia* that were partially responsible for the anti-inflammatory properties identified with this botanical. We hypothesized through the enrichment of an *E. angustifolia* fraction with chemically synthesized Bauer alkylamide 11 and Bauer ketone 23, that an elevated anti-inflammatory response would be achieved compared to the fraction itself, measured by the inhibition of prostaglandin E2 (PGE_2) and nitric oxide (NO) production by lipopolysaccharide (LPS) induced RAW264.7 mouse macrophage cells. Using microarray, qRT-PCR, western blots, and activity assays we set out to explain the molecular mode of action leading to the inhibition of PGE_2 production after treatments with fraction, enriched fraction, combination of Bauer alkylamide 11 and Bauer ketone 23, and these compounds individually. It was determined that the enriched fraction was capable of a more potent inhibition of LPS induced PGE_2 than the fraction alone, although NO production was not affected by either treatment. From the microarray analysis, no differentially expressed genes were identified with the treatments compared to the control after an eight hour treatment. Follow-up time-course qRT-PCR studies indicated a significant decrease in TNF-α and an increase of iNOS with a 24 hr incubation of treatment with the LPS induced RAW264.7 cells. Interestingly, LPS induced COX-2 protein was

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significantly increased by the fraction and Bauer ketone 23 individually. COX-2 activity was significantly decreased with all treatments after an eight hour incubation. In conclusion these studies provide evidence that the identified inhibition of PGE_2 production was due to the direct targeting of *Echinacea* products to the 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*). Several studies have been conducted to elucidate the cellular mechanism of action for the immune modulatory properties of *Echinacea* (*2-5*). 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 are found in neurons from the central and peripheral nervous system and concentrated in the brain, while CB2 receptors are mainly in immune cells, including macrophages (*6*). 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 (*7*). Endogenous ligands for the cannabinoid receptors include anandamide (AEA) and 2-arachidonyl glycerol (2-AG) sharing structural similarity with *Echinacea* alkylamides (*8*). Previous studies have determined that certain alkylamides have the ability to bind the CB2 receptor having Ki values around 60 nM with greater affinity than the natural ligands (*7*). The role of the CB2 receptor 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

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(TNF-α), was induced via the CB2 receptor by a standardized preparation of *Echinacea* called Echinaforce™ (*3*). This activity was attributed to certain alkylamides present in the Echinaforce™ preparation, including Bauer alkylamide 11 at 0.5 µM. Recently *Echinacea* ketones have been shown to have anti-tumorigenic and anti-inflammatory properties (*9, 10*). Therefore, Egger et al. set out to determine whether various ketoalkenes or ketoalkenynes of *E. pallida* could mediate their immune modulatory effects through the cannabinoid receptors, identifying no significant activity (*2*).

Significant inhibition of prostaglandin E2 (PGE_2) and nitric oxide (NO) production have been achievable with the treatment of *Echinacea* extracts, fractions, and pure constituents providing two excellent endpoints for the elucidation of species, as well as classes of compounds, that are important for the *in vitro* anti-inflammatory properties of *Echinacea* (*10-13*). Enzymes upstream of these endpoints, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), for PGE_2 and NO respectively, have been studied in order to further delineate how *Echinacea* modulates inflammation. Due to the involvement of the arachidonic acid cascade in the production of PGE_2 Muller-Jakic et al. determined that alkylamides isolated from an Soxhlet n-hexane extract of *E. angustifolia* were capable of inhibiting both cyclooxygenase and 5-lipoxygenase activity *in vitro* (*14*). Another study showed that certain alkylamides from a CO_2 extract of *E. angustifolia* abrogate COX-2 activity, but have no effect on COX-2 mRNA or protein in neuroglioma cells (*15*). Zhai et al. described the inhibition of NO identified with Soxhlet ethanol extracts of *E. angustifolia*, *E. pallida*, and *E. purpurea* was due to an inhibition of iNOS protein expression, attributing this effect to the lipophilic alkylamides (*13*).

The presence of Bauer alkylamide 11 at a concentration of 3.55 µM and Bauer ketone 23 at 0.83 µM of *E. angustifolia* fractions capable of inhibition of prostaglandin E2 was a key finding in our laboratory (*10*). During a re-fractionation of *E. angustifolia* we identified a first round fraction capable of significant PGE_2 inhibition, but to a lesser extent than

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previously identified in our prior studies. It was determined that the decreased 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 through enrichment of *Echinacea* products with certain constituents at the proper concentrations it may be possible to tailor this botanical to effectively target specific bioactivities. Our studies were conducted to examine how enrichment of an *E. angustifolia* fraction with Bauer alkylamide 11 and Bauer ketone 23, as well as these synthetic compounds in combination or individually influence PGE_2 and NO production in the RAW264.7 mouse macrophage cell line and further elucidate a mechanism of action leading to the modulation of these inflammatory endpoints.

Materials and Methods

Plant Material and Extraction

E. angustifolia (PI636395) root material was provided by the USDA North Central Regional Plant Introduction Station (NCRPIS, Ames, IA) and collected, stored, and Soxhlet ethanol extracted as previously described (*11*). 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.

Semi-Preparative HPLC Fractionation

The fractionation of a 95 % ethanol extract of *Echinacea angustifolia* was conducted using Semi-Preparative HPLC using methods to avoid endotoxin contamination as previously described (*10*).

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. (*10*).

Alkylamide and Ketone Synthesis

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Alkylamide and ketone synthesis was conducted as described previously accounting for percent purity when determining concentrations of compounds (*10, 11*).

Cell Culture

RAW264.7 mouse monocyte/macrophage cells were purchased from American Type Culture Collection (cat: TIB-71, Manassas, VA). The conditions under which the cells were cultured have been previously described (*11, 16*).

Echinacea angustifolia Fraction and Constituent Treatments

Five treatments were consistently used for each assay, which were: (1) *E. angustifolia* fraction 3 at 1 µg/ml; (2) enriched fraction 3 consisting of *E. angustifolia* fraction 3 at 1 µg/ml (which contained Bauer alkylamide 11 at a concentration of 0.05 µM), synthetic Bauer alkylamide 11 at 3.5 µM, and Bauer ketone 23 at 0.83 µM; (3) the combination of Bauer alkylamide 11 at 3.55 µM and Bauer ketone 23 at 0.83 µM; (4) chemically synthesized Bauer alkylamide 11 at 3.55 µM; and (5) chemically synthesized Bauer ketone 23 at 0.83 µM.

Measurement of Prostaglandin E_2, Nitric Oxide, and Cytotoxicity

The production of prostaglandin E_2 was assessed using a PGE_2 enzyme immunoassay (GE Biosciences; Piscataway, NJ) after treating RAW264.7 mouse macrophage cells for eight hours with fractions from *E. angustifolia* and with or without lipopolysaccharide (*E. coli* O26:B6, Sigma; St. Louis, MO) as previously described (*11*). Quercetin (3, 5, 7, 3'4'-pentahydroxy flavon), was chosen as the positive control for this assay due to its anti-inflammatory properties at a concentration of 10 µM (Sigma; St. Louis, MO).

Nitric oxide production was analyzed after a 24 hour incubation with *Echinacea* fraction or pure constituent using Griess Reagent System (Promega; Madison, WI) following the manufacturers protocol. The assay has been previously described using the RAW264.7 cell line and outlined in LaLone et al. (*10*).

Cytotoxicity was analyzed using the Celltiter96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) as previously described (*11*). Each

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fraction and compound was screened for cytotoxicity at the concentrations in the PGE_2 assay and incubated for 24 hours.

Western blots

RAW264.7 cells were grown in 10 cm Petri dishes to 80% confluency (overnight) and treated for eight hours. Cells were washed twice with ice cold 1X phosphate buffered saline (PBS) and lysed on ice for 5 minutes with 500 µl of lysis buffer (50 mM Tris-hydrochloride, 2mM ethylenediamine tetraacetic acid, 2 mM ethylene glycol tetraacetic acid, 150 mM sodium chloride, 2 mM phenylmethanesulphonylfluoride, 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°C to form 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 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. (*17*) and visualized using ECL detection. ImageQuaNT software was used for semi-quantitative analysis as previously described (*18*).

RNA extraction and DNAse digestion

RAW264.7 mouse macrophage cells were grown in 75cm flasks to 80% confluency and treated for eight hours 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 h, 1 h, 2 h, 4 h, 8 h, 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 washing flask or plate twice with PBS.

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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 22,600 probe sets, representing 14,500 well-substantiated genes (Affymetrix, Santa Clara, CA). The five treatments selected for the microarray analysis were the media + DMSO and media + DMSO + LPS controls, and *E. angustifolia* fraction 3 (1 µg/ml) + LPS, fraction 3 (1µg/ml) + synthetic Bauer alkylamide 11 (3.5 µM) + Bauer ketone 23 (0.83 µM) + LPS, and synthetic Bauer alkylamide 11 (3.55 µM) + Bauer ketone 23 (0.83 µM) + LPS. The controls were used to establish that the LPS effect was consistent with current literature. Our study was designed to determine differentially expressed (DE) genes important for the inhibition of LPS induced PGE_2 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 manufacturer (Affymetrix, Santa Clara, CA). The gene chips were ran 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 (BioRad, 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 GADPH using Primer3 (*19*) and procured from Integrated DNA Technologies, Inc. (Coralville, IA) (**Table 1**). Amplification conditions for the qRT-PCR

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were set at 95°C for 3 minutes, and 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, followed by 95°C for 1 minute, and 55°C for 1 minute.

Plasmid preparation for standard curves has been described previously (Hammer et al., unpublished), with the exception that PCR products were cloned into MAX Efficiency® DH5α™ Competent Cells.

COX activity assay

COX activity was measured using a COX Fluorescent Activity Assay Kit according to manufacturer's directions (Cayman Chemicals, Ann Arbor, MI) after an 8 hour treatment with *E. angustifolia* fraction 3, enriched fraction 3, 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 PGG_2 and 10-acetyl-3, 7-dihydroxyphenoxazine after the addition of arachidonic acid, which generates the fluorescent compound resorufin. The common plant flavanoid, quercetin, was used as a positive control at 25 µM.

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 µg/ml) and treated for twenty four hours. After the twenty four hour treatment the supernatant was collected for the assay. Supernatant was analyzed using the $MILLIPLEX_{MAP}$ Mouse Cytokine 32plex Panel following the manufacturer's protocol (Millipore, Billerica, MA).

Statistical Analysis

Both log transformed PGE_2 data and NO data were analyzed using randomized complete block design with variable levels of treatment, followed by a t-test based on pooled

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error variance to determine statistical significance compared to the (media + DMSO + LPS) control. In all figures, the data are represented as % of control ± standard error, normalizing the (media + DMSO + LPS) control to 100% PGE_2 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 ± 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).

The microarray experiment has a randomized complete block design with 4 replications as fixed block and 5 treatments (media + DMSO, media + 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 5 treatments. The raw data were normalized by the robust multi-array 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 the pairwise comparisons of interest were tested by unadjusted t-test. Differentially expressed genes were identified with false discovery rate (FDR) less than 0.0001% using Benjamini and Hochberg's method (1995).

Genes with significant treatment effects (FDR < 0.0001%) 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 *Echinacea angustifolia* Fraction

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Semi-preparative reversed phase HPLC was used to fractionate an extract of root material from *E. angustifolia* into five fractions and analyzed for their effect on LPS induced PGE_2 production in the RAW264.7 mouse macrophage cell line. The study led to the identification of three fractions that were capable of significantly inhibiting PGE_2 production (**Figure 1A**). Fraction 3, an alkylamide rich fraction equivalent in fractionation preparation to the *E. angustifolia* fraction 3 (inhibited PGE_2 to 1.1 ng/ml PGE_2) published by LaLone et al., showed potent PGE_2 reduction (inhibited PGE_2 to 1.8 ng/ml PGE_2) 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 ketone 23 (**Figure 1B**). From this analysis it was determined that Bauer alkylamide 11 was present at a concentration of 0.05 µM and there was no trace of Bauer ketone 23 in 1 µg/ml of fraction 3. Previously published studies conducted by our laboratory found that Bauer alkylamide 11 and ketone 23, at concentrations of 3.55 µM and 0.83 µM, respectively contributed to the activity of an earlier fraction prepared similar to the current fraction 3. Therefore, 3.5 µM of chemically synthesized Bauer alkylamide 11 and 0.83 µM of Bauer ketone 23 were added to 1 µg/ml of fraction 3 to produce an enriched fraction 3. *Echinacea angustifolia* fraction 3, enriched fraction 3, and combinations of individual constituents and fraction 3, were evaluated after an eight hour treatment with the RAW264.7 cells for PGE_2 and NO production, showing significant PGE_2 inhibition with all treatments and significant NO inhibition with the combination of chemically synthesized Bauer alkylamide 11 and ketone 23 and each constituent individually (**Figure 2**). The enriched fraction 3 was found to have significantly greater PGE_2 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 and Protein Studies with *E. angustifolia* Fraction and Constituents

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Gene expression was analyzed with GeneChip® Mouse Genome 430A 2.0 Arrays in order to identify target genes and determine pathways leading to the inhibition of PGE_2 production in the RAW264.7 mouse macrophage cells with selected treatments from **Figure 2** treated for eight hours. The media + DMSO control was compared to the media + DMSO + LPS control in order to establish that the expected genes were differentially expressed. With a false discovery rate of 0.0001% there were 3,257 differentially expressed (DE) genes were identified between the controls, which corresponded with the DE genes identified by Hammer et al. (unpublished). Of these 3,257 DE genes, 1,253 had increased expression levels (5.5% of total probe-sets) and 2,004 (8.9% of total probe-sets) had decreased expression levels, with 731 genes decreased at least 50% below the expression level of the media + DMSO control and 951 genes increased at least 50% above the expression level of media + 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 the immune response, cell death, and cell motility.

When searching for DE genes between the media + 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 (*20*) 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 media + 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.

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In order 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 was not significantly different throughout the time course from 0.5 hr to 24 hr (**Figure 4B**). The only significant increase in COX-2 gene expression was with the LPS treated samples compared to the media + DMSO control. TNF-α gene expression was significantly increased with treatment of the RAW264.7 cells with Bauer ketone 23 at the 0.5 hr time point compared to the media + DMSO + LPS control (**Figure 4C and 4E**). At the 1 hr time point TNF-α gene expression was significantly decreased with treatment of Bauer alkylamide 11 (**Figure 4C and 4E**). When gene expression analysis for TNF-α was carried out to 24 hr a significant decrease in gene expression was identified for *E. angustifolia* fraction 3 + LPS, enriched fraction 3 + LPS, combination of Bauer alkylamide 11 and Bauer ketone 23 + LPS as well as with each individual constituent + LPS compared to the media + DMSO + LPS control (**Figure 4C and 4E**). Treatments were also carried out without LPS showing no significant difference in TNF-α gene expression compared to the media + DMSO control (p-values >0.08). Lipopolysaccharide induced TNF-α protein production was also analyzed, indicating that each treatment + LPS, except for synthetic Bauer ketone 23 + LPS, were capable of significant inhibition (**Figure 5)**. A significant difference in iNOS gene expression was identified at the 24 hr time point, indicating an increase in iNOS mRNA with the enriched fraction 3 compared to the media + DMSO + LPS control (**Figure 4D and 4F**). The expected LPS induction of the RAW264.7 macrophage cells was identified with the increase of COX-2, TNF-α, and iNOS genes after treatment with the media + DMSO + LPS control compared to the media + DMSO control. GADPH was selected as the house keeping gene for the experiments, indicating no change in mRNA levels with any of the

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treatments during the time course from 0.5 hr to 24 hr (p-values >0.14). COX-1 mRNA was shown to increase at the 24 hr time point after treatment with the *E. angustifolia* fraction 3, enriched fraction 3, Bauer alkylamide 11, and Bauer ketone 23 compared to the media + DMSO + LPS control (p-values <0.02), 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 5A & B**), Bauer alkylamide 11(**Figure 6A & B**), and Bauer ketone23 (**Figure 7A & B**). From these analyses it was shown that there was a significant increase in COX-2 protein with fraction 3 at 1 µg/ml and 5 µg/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 µg/ml, yet α-tubulin protein level remained unchanged. Protein levels of lipopolysaccharide 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 ± 21.7% of control) and media + DMSO + LPS control after a 24 hr treatment .

Inhibition of Cyclooxygenase-2 Activity

COX-2 activity was analyzed after treating the RAW264.7 macrophage cells for eight hours 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. No significant differences in COX-2 activity were identified between any of the treatments without LPS compared to the media + DMSO control. When analyzing all LPS treated samples compared to the media + DMSO + LPS control the *E. angustifolia* fraction 3 (31.6 ± 10% of control), enriched fraction 3 (29.6 ±7.3% of control), and the combination of synthetic compounds (45.2 ± 12.3% of control) potently inhibited COX-2 activity with p-

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values less than 0.001 and synthetic Bauer alkylamide 11 (74.3 ± 11.0% of control) and synthetic Bauer ketone 23 (65.3 ± 16.3% of control) individually significantly inhibited COX-2 activity with –p-values less than 0.05.

Discussion

A major finding in this study is that by enriching an *E. angustifolia* fraction (1 µg/ml) with Bauer alkylamide 11 (3.5 µM) and Bauer ketone 23 (0.83 µM) a more potent inhibition of the LPS induced inflammatory mediator, PGE_2, was identified compared to that seen with the fraction alone after an eight hour treatment on the RAW264.7 macrophage cells. From these results, it appears that Bauer alkylamide 11 and Bauer ketone 23 partially accounted for the PGE_2 inhibitory capabilities of the fraction, but do not show evidence of additivity. This finding indicates that it is possible to manipulate an *Echinacea* preparation to target a specific bioactivity after discovering constituents of importance as well as the concentration required to elicit the desired effect. Having the ability to target specific bioactivities with *Echinacea* preparations would be very important in the development of botanical products that are used for specific medicinal purposes. Results collected on 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 (*21, 22*). In order to characterize which constituents are necessary for particular medicinal outcomes and at what concentrations, more 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 PGE_2 production in the RAW264.7 cells that was previously identified with certain *Echinacea* fractions (*10, 11*). Using microarray analysis and qRT-PCR it became apparent that our *Echinacea* treatments were not acting on the gene expression level for COX-2. Also, western blot analysis led to the intriguing finding that *E. angustifolia* fraction 3 and Bauer

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ketone 23 could increase COX-2 protein levels after an eight hour treatment, yet also have the ability to inhibit PGE_2 production at that same time point. These studies directed the way to another key observation, which is supported by previous literature (*15*) that the identified inhibition of LPS induced PGE_2 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 (*23*). 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 its possible usefulness as an anti-inflammatory agent. The results obtained from this study allow us to hypothesize a model for the inhibition of PGE_2 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* alkylamides and ketones 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. Therefore, we would observe a decrease in COX-2 activity, a decrease in PGE_2 production, and an increase in COX-2 protein, with no change in gene expression, which is consistent with our findings.

Tumor necrosis factor alpha has been shown to be 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 Echinaforce™ modulated TNF-α gene expression in human monocytes/macrophages (*3*). The results from this study show that LPS induced TNF-α

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mRNA decreases around 10 hours after treatment of the alkylamide rich preparation, with a steady decline until approximately 25 hours. Other findings from this study indicate 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 µM (3). Our qRT-PCR time-course was carried out to 24 hours 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 with the individual constituents, corresponding with the findings of Gertsch et al., although the concentration of alkylamide in our studies was slightly higher at 3.55 µM. Our studies indicate that these treatments, excluding Bauer ketone 23, also significantly inhibit 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 hour time point. An interesting finding from our studies was that Bauer ketone 23 (0.83 µM) did not significantly modulate TNF-α protein levels. Egger et al. determined that ketones, analyzed at 3% (v/v) DMSO do not appear to mediate their immunomodulatory effects through the cannabinoid receptors (*2*) and therefore it is likely that Bauer ketone 23 acts through a different mechanism to inhibit the noted TNF-α gene expression.

Lipopolysaccharide induced NO production was shown to be significantly inhibited by treatments of synthetic Bauer alkylamide 11 and ketone 23, Bauer alkylamide 11 individually, and Bauer ketone 23 individually after a 24 hour 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 media + 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 induce iNOS protein levels after a 24 hour incubation. Previous studies on *Echinacea* extract treated

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RAW264.7 cells incubated for 23 hours determined that the extracts were capable of NO inhibition, not through direct scavenging of the free radical, but through the inhibition of iNOS (*13*). Chen et al. also demonstrated that alkylamides were capable of inhibiting LPS induced NO production after a 24 hour incubation in the RAW264.7 cell line, including Bauer alkylamide 11, with an ID_{50} of 23.9 µM (*12*). Our prior studies had also indicated that Bauer ketones could significantly inhibit NO production at concentrations as low as 1 µM (*10*). 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.

The results acquired from this study provide evidence that through the manipulation 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 additivity was identified with treatments that combined synthetic Bauer alkylamide 11 and Bauer ketone 23 in the PGE_2 screening, although their addition to the *E. angustifolia* fraction proved to increase anti-inflammatory potential. The results obtained from our COX-2 activity studies indicated that 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 PGE_2 production.

Abbreviations Used

E. = *Echinacea*

PI = Plant Introduction

DMSO = Dimethyl Sulfoxide

PGE_2 = Prostaglandin E2

NO = Nitric Oxide

LPS = Lipopolysaccharide

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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.

Acknowledgment

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Figure Legends

Figure 1. A. Fraction 3 from a 2009 extract of *Echinacea angustifolia* (PI631293) significantly inhibited PGE_2 production in RAW264.7 cells. The black bars represent PGE_2 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 % (4.3 ng/ml). Treatments were also performed without LPS induction showing significant reduction in PGE_2 production with fractions 2, 3, and 5 ($p < 0.04$). The treatments without LPS were compared to the media + DMSO control set at 100 % (0.03 ng/ml). * 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 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 fractions 3 with peaks whose 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 µM.

Figure 2. Lipopolysaccharide induced PGE_2 and NO production in RAW264.7 cells treated with *E. angustifolia* fraction, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and ketones 23. The black bars represent PGE_2 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 media + DMSO + LPS control that was set at 100 % PGE_2 production (2.5 ng/ml) and NO production (18.8 ng/ml). The treatments without LPS were compared to the media + DMSO control set at 100 % PGE_2 production (0.05 ng/ml) and NO production (~0 ng/ml) identifying no significant differences with treatment for either endpoint. Quercetin was used as a positive control for both studies and showed significant inhibition of

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PGE_2 and NO production ($p<0.0001$). * 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 any of the treatments or combination of treatments (data not shown).

Figure 3. A. Hierarchical cluster analysis of differentially expressed genes in RAW264.7 mouse macrophages. 2,719 differentially expressed genes were identified comparing the media + DMSO control to the media + 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 = Media + DMSO control, M1 = Media + 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 size of each cluster is given in parentheses on the right above the cluster graph.

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 $p<0.001$. 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 $p<0.001$. E. qRT-PCR analysis at time points with significant treatment effects for TNF-α gene when compared to the media + DMSO + LPS control. * and ** are representative of $p<0.05$ and

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$p<0.001$. Bars represent the mean ± standard error. F. qRT-PCR analysis at time point with significant treatment effect for iNOS gene when compared to the media + DMSO + LPS control. * and ** are representative of $p<0.05$ and $p<0.001$. Bars represent the mean ± standard error.

Figure 5. Lipopolysaccharide induced TNF-α production in RAW264.7 cells treated with *E. angustifolia* fraction, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and ketones 23. The light grey bars represent TNF-α levels after treatment with *Echinacea* fraction, enriched fraction or compounds. The dark grey bars represent TNF-α levels after induction with 1 μg/ml LPS and treatment (n = 3). All treatments + LPS were compared to media + DMSO + LPS control. The treatments without LPS were compared to the media + DMSO control ($p<0.0001$). * and ** are representative of $p<0.05$ and $p<0.001$. Each bar represents mean ± 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 ketone 23. N=3 for each blot. B. Semi-quantitative representation of the blots from figure 6A. Bars represent mean percent of media + DMSO + LPS control ± standard error. Lipopolysaccharide induced COX-2 protein from 24.3 ± 8.2% average for the media + DMSO control to 100 ± 20.6% average for the media + DMSO + LPS control. There was no significant LPS affect for the media + DMSO control with COX-1 (96.9 ± 1.8% of control average) or α-tubulin (104.4 ± 3.4% of control average) on protein level compared to the media + DMSO + LPS control. Quercetin was used as a positive control at 100 μM for the reduction of LPS induced COX-2 protein 41.3 ± 16.9% of control. Quercetin did not significantly affect LPS induced COX-1 (85.3 ± 3.2% of control average) or α-tubulin (109.6 ± 16.7 % of control average). * and ** are representative of $p<0.05$ and $p<0.001$ when compared to media + DMSO + LPS control.

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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. Semi-quantitative representation of the blots from figure 7A. Bars represent mean percent of media + DMSO + LPS control ± standard error. Lipopolysaccharide induced COX-2 protein from 19.1 ± 6.2% average for the media + DMSO control to 100 ± 10.9% average for the media + DMSO + LPS control. There was no significant LPS affect for the media + DMSO control with COX-1 (83.4 ± 3.0% of control average) or α-tubulin (94.7 ± 2.1% of control average) on protein level compared to the media + DMSO + LPS control. Quercetin was used as a positive control at 100 µM for the reduction of LPS induced COX-2 protein 71.4 ± 3.1% of control. Quercetin did not significantly affect LPS induced COX-1 (96.1 ± 6.5% of control average) or α-tubulin (98.0 ± 4.3 of control average). * and ** are representative of $p<0.05$ and $p<0.001$ when compared to media + DMSO + LPS control.

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 figure 8A. Bars represent mean percent of media + DMSO + LPS control ± standard error. Lipopolysaccharide induced COX-2 protein from 4.8 ± 2.8% average for the media + DMSO control to 100 ± 12.0% average for the media + DMSO + LPS control. There was no significant LPS affect for the media + DMSO control with COX-1 (95.4 ± 3.3% of control average) or α-tubulin (103.9 ± 7.3% of control average) on protein level compared to the media + DMSO + LPS control. Quercetin was used as a positive control at 100 µM for the reduction of LPS induced COX-2 protein 53.0 ± 15.7% of control. Quercetin did not significantly affect LPS induced COX-1 (95.5 ± 2.2% of control average) or α-tubulin (91.8 ± 9.7 of control average). * and ** are representative of $p<0.05$ and $p<0.001$ when compared to media + DMSO + LPS control.

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Figure 9. Lipopolysaccharide induced COX-2 activity in RAW264.7 cells treated with *E. angustifolia* fraction, enhanced fraction, and chemically synthesized Bauer alkylamide 11 and ketones 23. The black bars represent COX-2 activity levels after induction with 1 µg/ml LPS and treatment (n = 3). All treatments + LPS were compared to media + DMSO + LPS control that was set at 100 % COX-2 activity (5.8 nmol/min/ml). The treatments without LPS were compared to the media + 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 $p<0.001$. Each bar represents % of control ± standard error.

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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	GCTCGGCTTCCAGTATTGAG
TNF-α	S	AGGAGGGAGAACAGAAACTC
	AS	AATGAGAAGAGGCTGAGACA
iNOS	S	GTCTTGGTGAAAGTGGTGTT
	AS	GTGCTTGCCTTATACTGGTC
GAPDH	S	CAATGTGTCCGTCGTGGAT
	AS	AGCCCAAGATGCCCTTCAG

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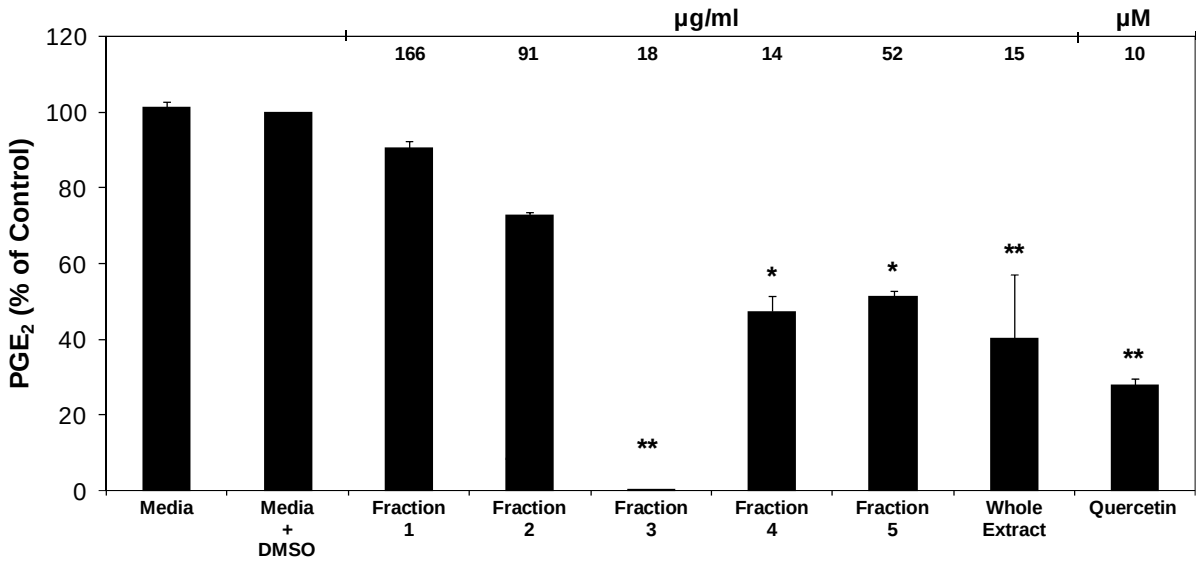
Figure 1.

A



B

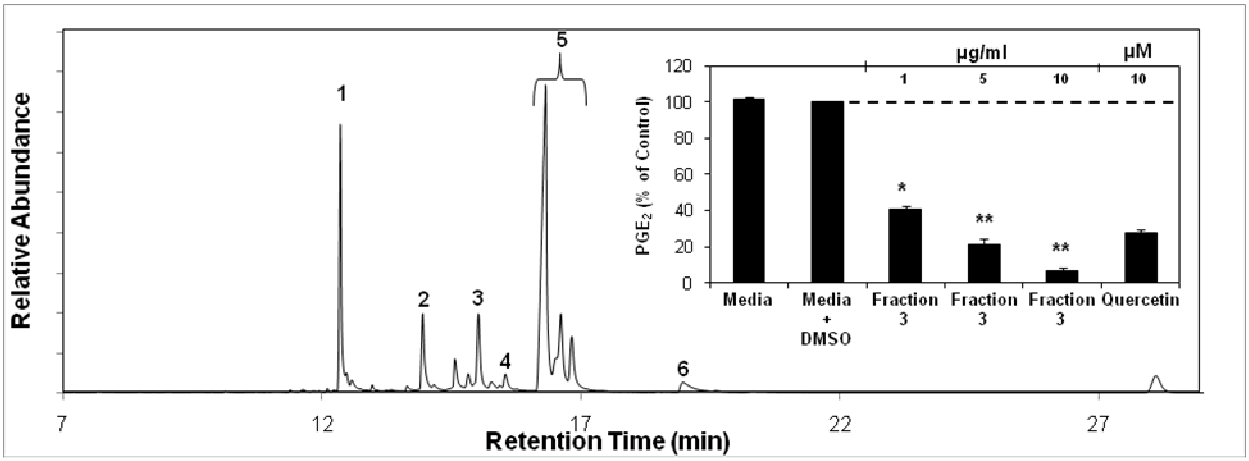


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Figure 2.

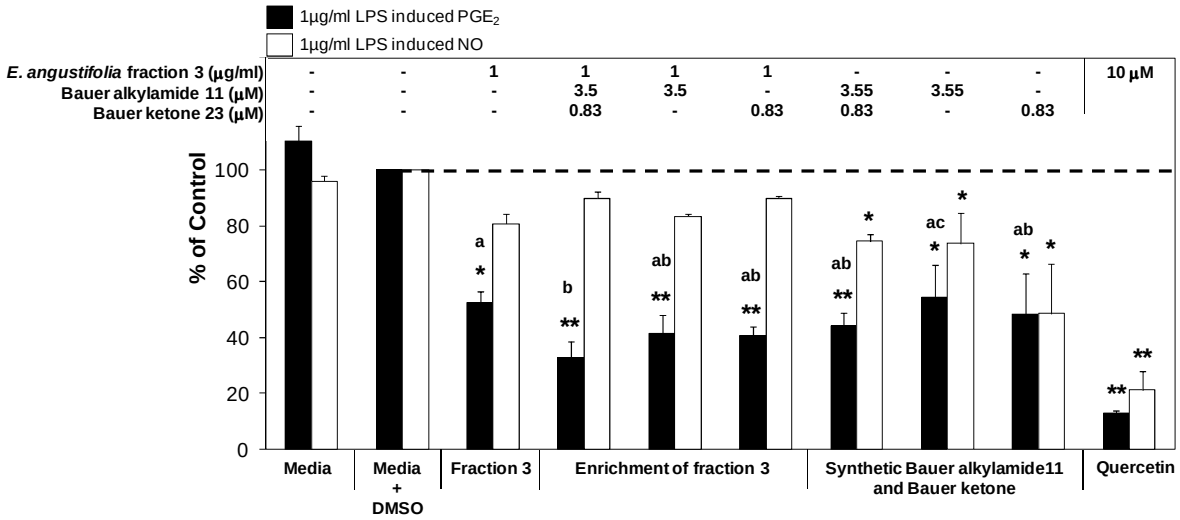

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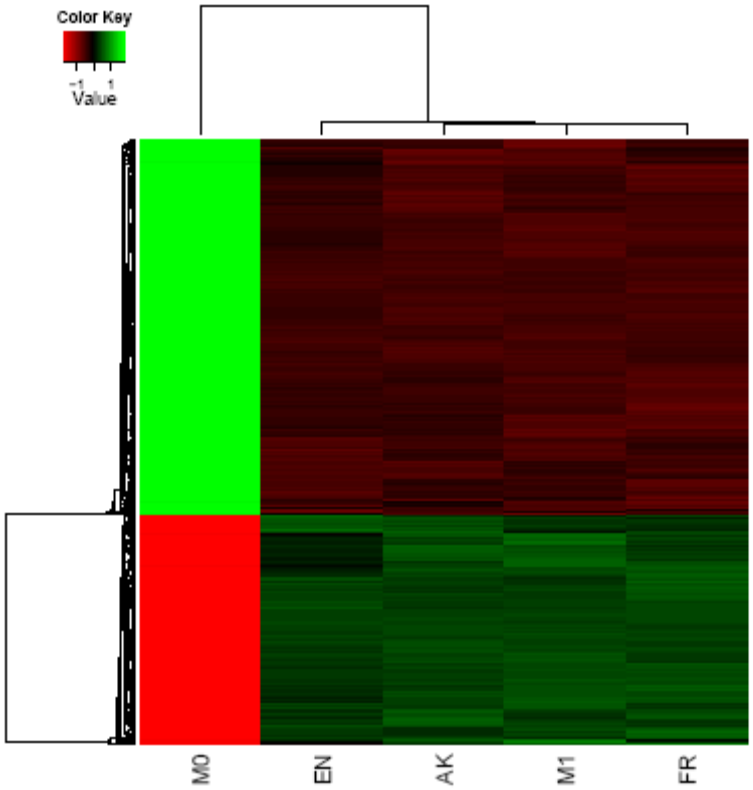
Figure 3.

A.



B.

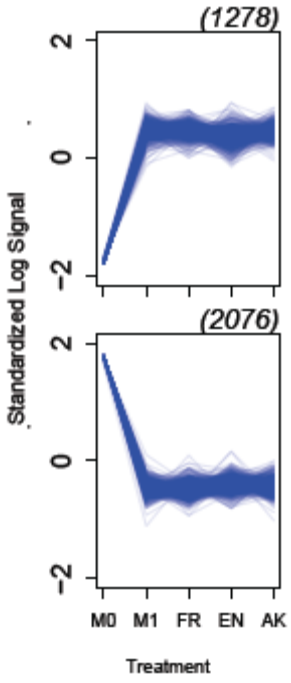


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Figure 4.

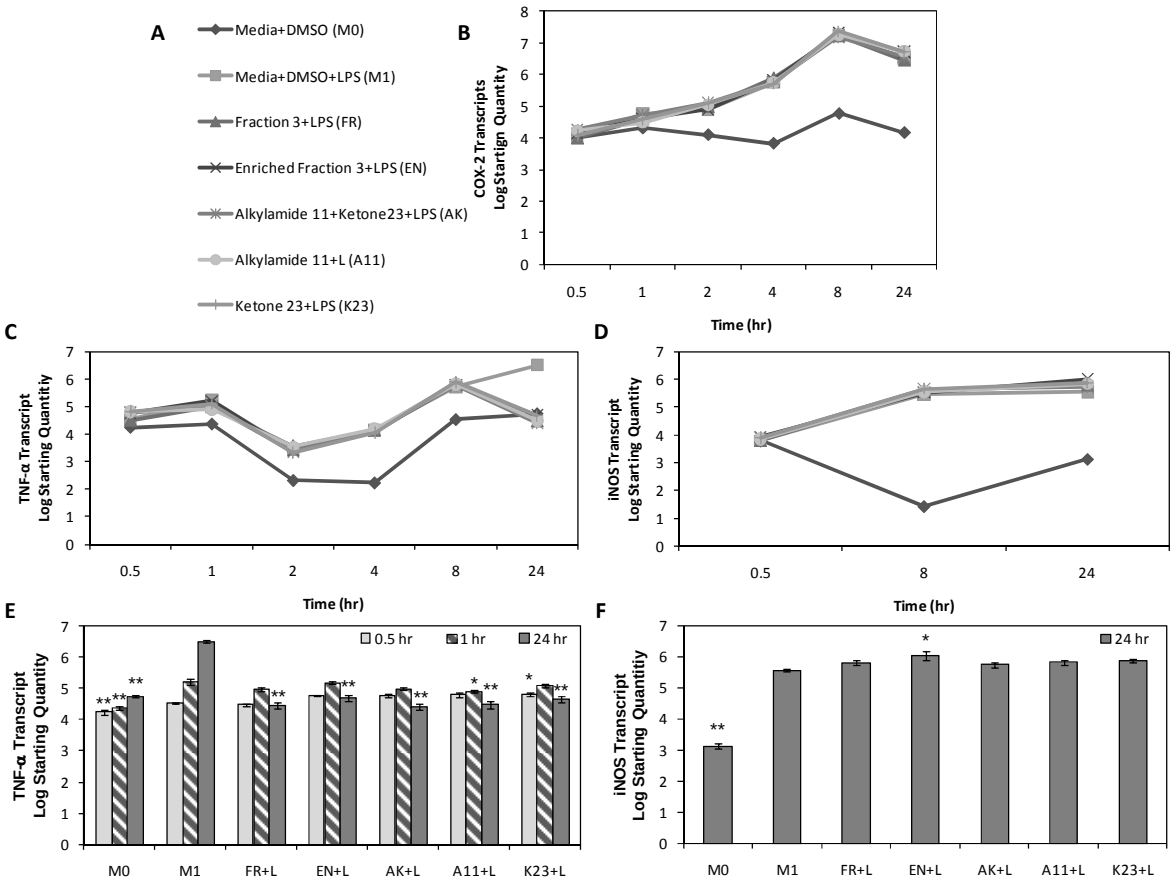

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Figure 5.

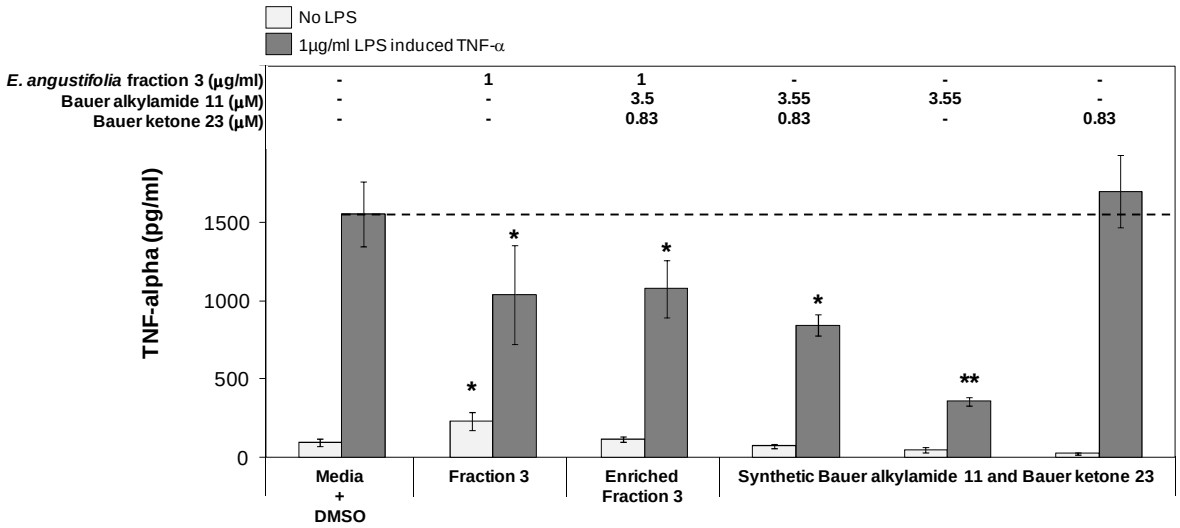

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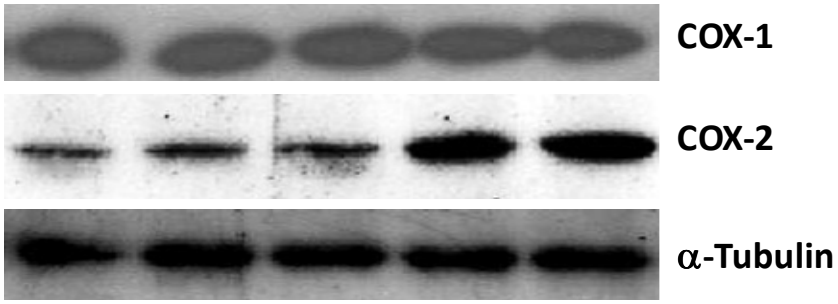
Figure 6.

A

Media + DMSO	**+**	**-**	**-**	**-**	**-**
***E. angustifolia* fraction 3**	**-**	**-**	**1 µg/ml**	**1 µg/ml**	**5 µg/ml**
Bauer alkylamide 11	**-**	**3.55 µM**	**3.5 µM**	**-**	**-**
Bauer ketone 23	**-**	**0.83 µM**	**0.83 µM**	**-**	**-**



B

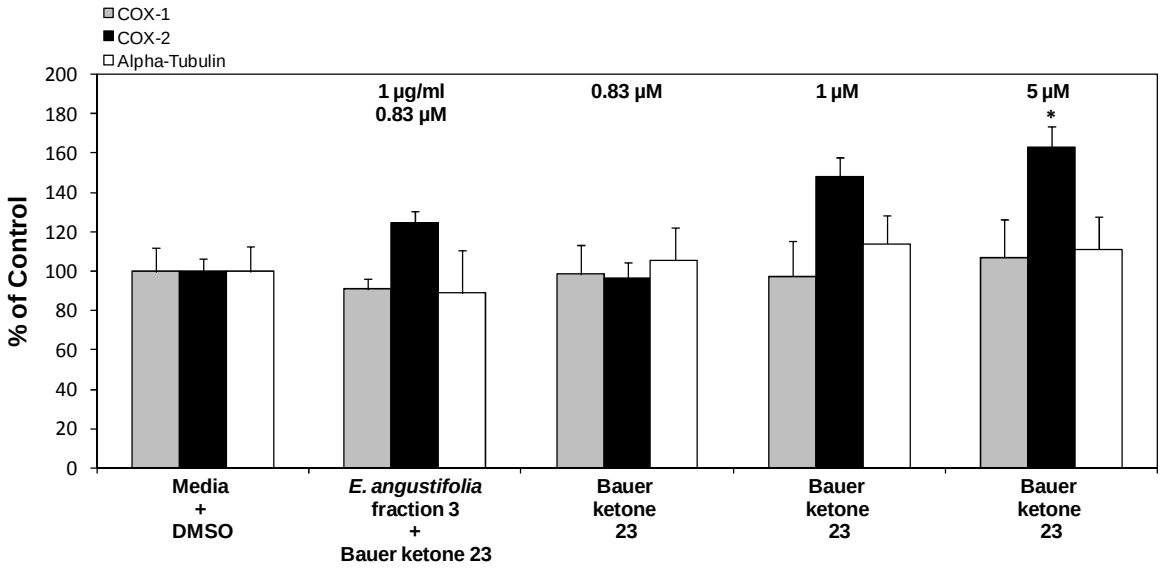


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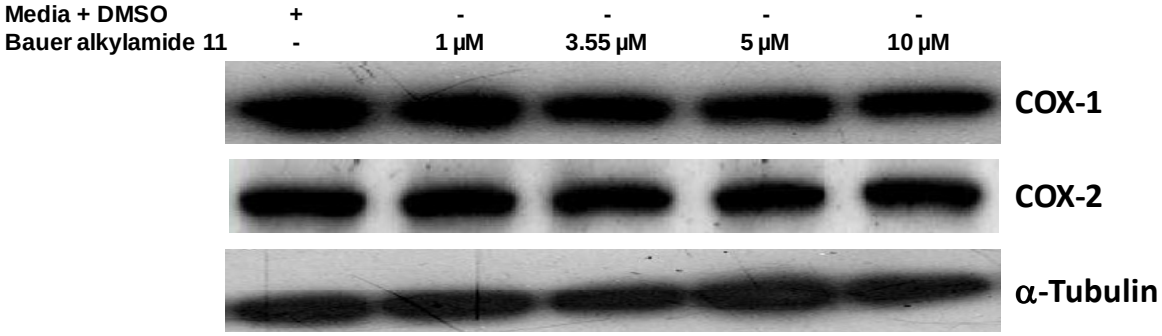
Figure 7

A



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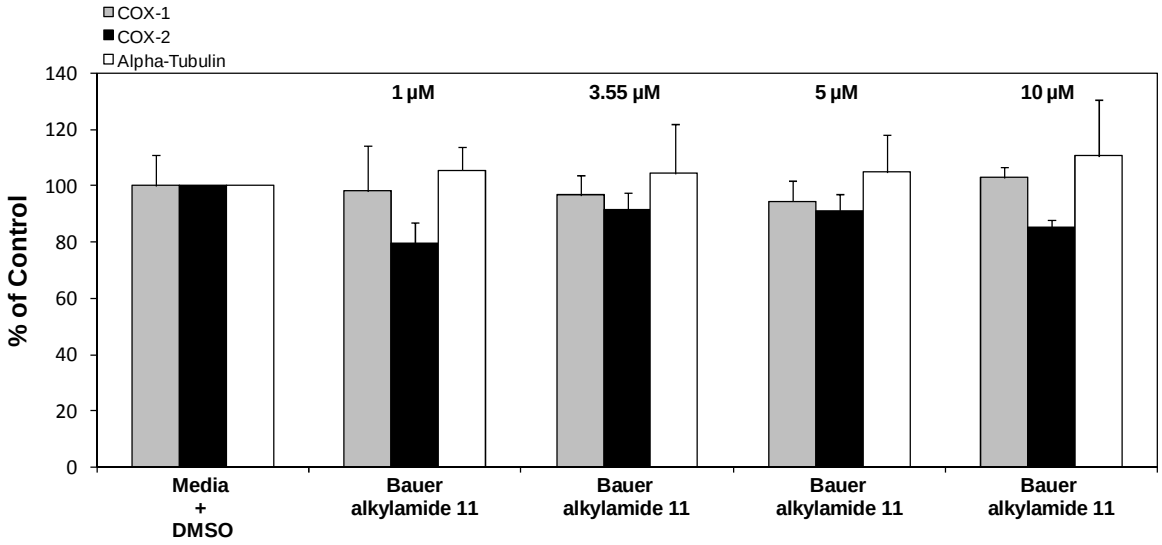

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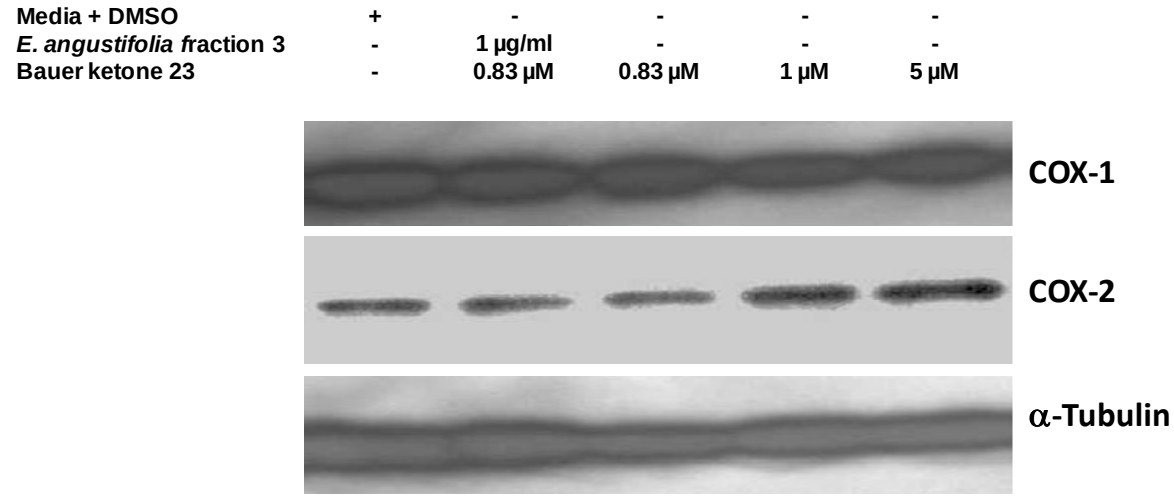
Figure 8.

A



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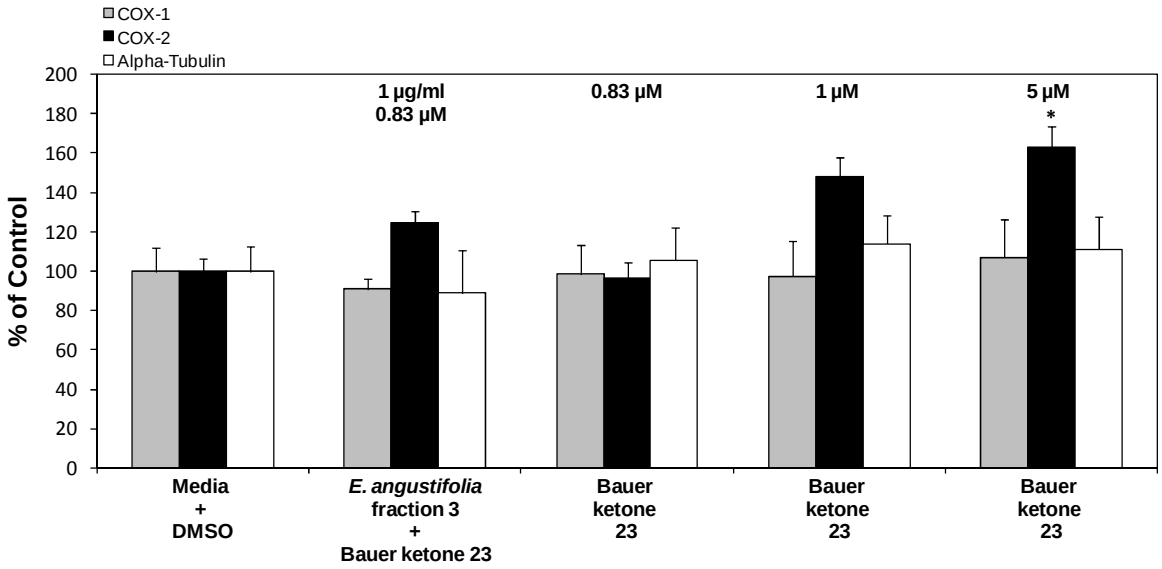

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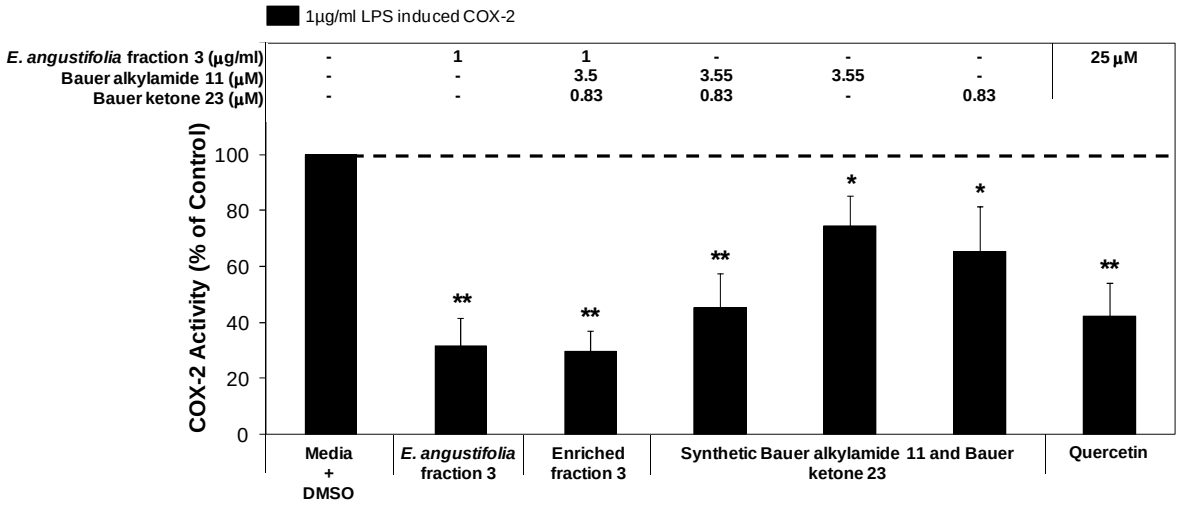
Figure 9.



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CHAPTER 6: GENERAL CONCLUSIONS

Importance of Botanical Research

For centuries plant preparations have been used medicinally to treat a wide variety of human diseases and ailments. Approximately 400 years ago Indian tribes used *Echinacea angustifolia, Echinacea purpurea,* and *Echinacea pallida* plants to treat anything from horse saddle sores to rheumatism *(1).* *Echinacea* is a plant that has found its way to modern use, commonly taken for the treatment of the common cold and other upper respiratory infections. The popularity of this botanical has been consistent throughout the last decade with estimated annual sales in the multi-million dollar range *(2)*. The importance of research regarding the safety and efficacy of the botanical *Echinacea* cannot be understated, due to the large quantity of people that consume such preparations. Research on this botanical has provided evidence that *Echinacea* has anti-viral, anti-oxidant, anti-microbial, anti-tumorigenic, and anti-inflammatory properties *(3-5)*. Many of these bioactivities have been attributed to certain classes of constituents of *Echinacea*, with a strong focus on the major class of lipophilic compounds, alkylamides.

Goals of the Studies Presented in this Dissertation

The overarching goal of the studies presented in this dissertation was to strengthen the knowledge and understanding of the anti-inflammatory potential of the botanical *Echinacea*. Our research set out to identify *Echinacea* constituents that are key modulators of the immune system through the use of a mouse macrophage model system and determine the mechanistic basis for the identified modulation.

Advancing the Field of *Echinacea* Research

The research conducted in this dissertation advances the field of *Echinacea* botanical research by identifying *Echinacea* species that have the strongest potential for use as anti-inflammatory products and establishes a basis to examine alkylamides and ketones at

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concentrations relevant to those found in the plant for their ability to interfere with the inflammatory response. All bioactivity studies conducted with *Echinacea* treatments were paralleled with screens for cytotoxicity, therefore also examining the safety of this botanical. Our studies were the first to credit ketones with the ability to inhibit inflammatory endpoints *in vitro*. Further, this research leads to a proposed mechanism for the inhibition of PGE_2 production by selected *Echinacea angustifolia* treatments.

Summary of Research conducted in this Dissertation and Major Findings

Our research used the well established RAW264.7 mouse macrophage cell line. Lipopolysaccharide, a common endotoxin, was applied to the cells to mimic the initiation of an *in vitro* inflammatory response. Through the activation of the arachidonic cascade, a key lipid mediator of inflammation, prostaglandin E2 (PGE_2), was produced. To initiate our studies for this dissertation on the anti-inflammatory properties of *Echinacea*, root material from six of the nine species of *Echinacea* were Soxhlet ethanol extracted and analyzed for their ability to modulate PGE_2 production. It was determined that *E. angustifolia, E. pallida, E. sanguinea,* and *E. simulata* significantly inhibited the production of PGE_2 at a concentration of 15 µg/ml. These findings were interesting in that *E. purpurea* was incapable of inhibiting this inflammatory endpoint at 15 µg/ml, but is consistently found in commercial preparations of this botanical supplement. Examination of the known classes of constituents through HPLC analysis indicated that the amount of alkylamides, caffeic acid derivatives, and ketones vary significantly among the bioactive *Echinacea* species and therefore provide a rational for thorough investigation of each class of constituent individually to better understand the species data. Parallel cytotoxicity screens established that high concentrations of *Echinacea* extracts, >100 µg/ml were required to cause macrophage cell death, indicating the safety of this herb and discounting cytotoxicity as a factor leading to PGE_2 inhibition. It is of importance to note that our studies revealed little

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variability in bioactivity results between repeat extractions or harvest years, allowing for consistency between replications and studies. Another key finding from this study was that alkylamides were consistent inhibitors of PGE_2, although not at the low concentrations identified in the species. This data led to the hypothesis that no single compound can account for the PGE_2 inhibition noted with the *Echinacea* extracts and therefore the anti-inflammatory alkylamides must be acting either synergistically or additively with each other or other compounds. Finally, these initial studies indicated that the *Echinacea* alkylamides were not cytotoxic to the RAW264.7 macrophage cells, again providing evidence of safety for the use of this botanical.

In order to identify which *Echinacea* constituents were key contributors to the inhibition of PGE_2 production seen with the *Echinacea* extracts, semi-preparative reverse-phased HPLC fractionation was applied to *E. angustifolia*, *E. pallida*, *E. purpurea*, and *E. tennesseensis* extracts. This method allowed for the separation of compounds according to their hydrophobic properties, with caffeic acid derivatives eluting in the first fractions and alkylamides and ketones following in the middle and later fractions. The fractionation studies were used to test the hypothesis that alkylamides acted in a synergistic or additive manner with other compounds to elicit their *in vitro* anti-inflammatory properties. Through the fractionation of *E. pallida*, it was determined that ketones were an important class of constituents capable of inhibiting PGE_2 and nitric oxide (NO) production in the RAW264.7 mouse macrophage model. For these studies NO was another endpoint chosen for the study of the anti-inflammatory properties of *Echinacea*, due to its important role during inflammation. NO was screened after 24 hr treatments with *Echinacea* fractions or constituents, which allowed us to follow the inflammatory response induced by LPS out to a later time point. The analysis of the ketones provided evidence that Bauer ketone 23 and 24 were of importance for the identified PGE_2 inhibitory capabilities of *E. pallida*. These

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studies were the first to look for anti-inflammatory potential with the ketones of *Echinacea* and identify this class of compounds as inflammatory modulators. As found with the extracts and alkylamides, significant cytotoxicity was not identified for any of the Bauer ketones screened. It was determined through the fractionation of all four species that fractions containing caffeic acid derivatives were unable to inhibit PGE_2, even at concentrations greater than 100 µg/ml. In our search for compounds that act synergistically or additively we tried combinations of constituents found in second round fractions of *E. angustifolia*, Bauer alkylamide 10 and 11. At the concentrations identified to be present in the second round fraction, the combination of Bauer alkylamide 10 and 11 was unable to inhibit PGE_2, although the individual constituents at the same concentrations were significantly inhibitory. The most significant findings from the fractionation studies were that synthetic Bauer alkylamide 11 at a concentration of 3.55 µM and Bauer ketone 23 at a concentration of 0.83 µM, which were concentrations corresponding to what was found in the fractions, were able to partially explain the inhibition of PGE_2 identified with their respective third round *E. angustifolia* fractions. The results from this study provided no evidence of additivity or synergy between the compounds for the inhibition of PGE_2 or NO production and indicated that other, unidentified constituents play a key role in the anti-inflammatory activity of *Echinacea*. It was interesting to find constituents that could modulate a bioactivity at the concentrations found in the plant preparation.

From the discovery of the importance of Bauer alkylamide 11 and Bauer ketone 23 for the inhibition of the inflammatory mediator PGE_2 and the understanding of the concentrations that were effective for this outcome, we re-fractionated an *E. angustifolia* extract to produce an alkylamide rich first round fraction with similar chemical composition and PGE_2 inhibition capabilities as we had found in our earlier studies. Upon repeating the fractionation protocol used previously, we found a fraction capable of significant PGE_2

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inhibition, although to a lesser extent than what was observed in our initial fractionation studies. GC-MS analysis identified that Bauer alkylamide 11 was present at a concentration of 0.05 µM and Bauer ketone 23 was not detected in the newly prepared *E. angustifolia* fraction. We then hypothesized that enrichment of the *E. angustifolia* fraction with synthetic Bauer alkylamide 11 to a concentration of 3.55 µM and Bauer ketone 23 to a concentration of 0.83 µM would increase the anti-inflammatory potential compared to the fraction alone. The hypothesis was correct, with the enriched fraction showing the ability to inhibit PGE_2 to a more significant extent than the unmodified fraction. Therefore this study provided evidence that it is indeed possible to manipulate an *Echinacea* fraction to act on a specific bioactivity when constituents and concentrations of importance have already been determined, which could pave the way for medicinal applications that can target specific symptoms. Mechanistic studies were then undertaken to understand how treatments of *E. angustifolia* fraction, enriched fraction, combination of synthetic Bauer alkylamide 11 and ketone 23, and these constituents individually inhibit PGE_2 production. Because LPS induced PGE_2 is produced through the arachidonic acid cascade by the action of the COX-2 enzyme, COX-2 gene expression, protein expression, and activity were studied after treatments in the mouse macrophage cell line. It was determined that LPS induced COX-2 gene expression was not affected by treatment over a 24 hr time course, but protein levels were increased significantly with the treatment of *E. angustifolia* fraction 3 + LPS and Bauer ketone 23 + LPS at 8 hours, consistent with the time point for the PGE_2 data in the RAW264.7 macrophages. The results from the LPS induced COX-2 activity and PGE_2 studies indicate that all treatments of *Echinacea* fraction, enriched fraction, and constituents inhibit the enzymatic activity and PGE_2 production. These findings have led to the proposal of a model as to how the treatments inhibit PGE_2 production, yet show an increase in COX-2 protein levels (**Figure 1**).

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To fully understand the model being proposed it is important to understand how our cell line responds to LPS under ordinary conditions. During a normal response in the RAW264.7 mouse macrophage cells to lipopolysaccharide, arachidonic acid (AA) is released from the phospholipid membrane by phospholipase A2. Meanwhile LPS binds to lipopolysaccharide binding protein (LBP) and toll like receptor 4 (TLR4) activating the NF-kB signal transduction cascade leading to the expression of COX-2 mRNA, which is then translated into the COX-2 enzyme that converts AA to prostaglandin G2 (PGG_2). PGG_2 is then converted to an eicosanoid, possibly PGE_2. It has previously been demonstrated that COX-2 is degraded by the ubiquitin (Ub) proteasome system *(6)*. COX-2 is polyubiquitinated by Ub ligases, which allows the enzyme to be recognized by the 26S proteosome and rapidly degraded. Ubiquitination occurs on lysine residues of target proteins where an isopeptide bond is created between the lysine and the C-terminal glycine of ubiquitin *(7)*. The model described in **Figure 1** indicates that LPS stimulates the production of COX-2 enzyme and AA is released from the phospholipid membrane, but AA is unable to bind to the active site of COX-2. It is proposed in the model that the inability of AA to bind to the enzyme could be due to the competitive or non-competitive binding of *Echinacea* fraction or constituent, which blocks AA. Alternatively, the *Echinacea* product in the model could be directly binding to AA making it too large to enter the active site of the enzyme. This would cause an inhibition of enzyme activity, a decrease in the amount of AA allowed to bind to the COX-2 enzyme, and therefore an inhibition of PGE_2 production. It is also proposed in **Figure 1** that the *Echinacea* fraction or ketone interfere with ubiquitination of the COX-2 enzyme, either by directly blocking ubiquitination sites, by interfering with the ubiquitin ligase, or by causing a conformation change of the enzyme which makes the ubiquitination sites inaccessible after binding to the active site. Due to the proposed interference of ubiquitination in the model the COX-2 protein levels would increase because

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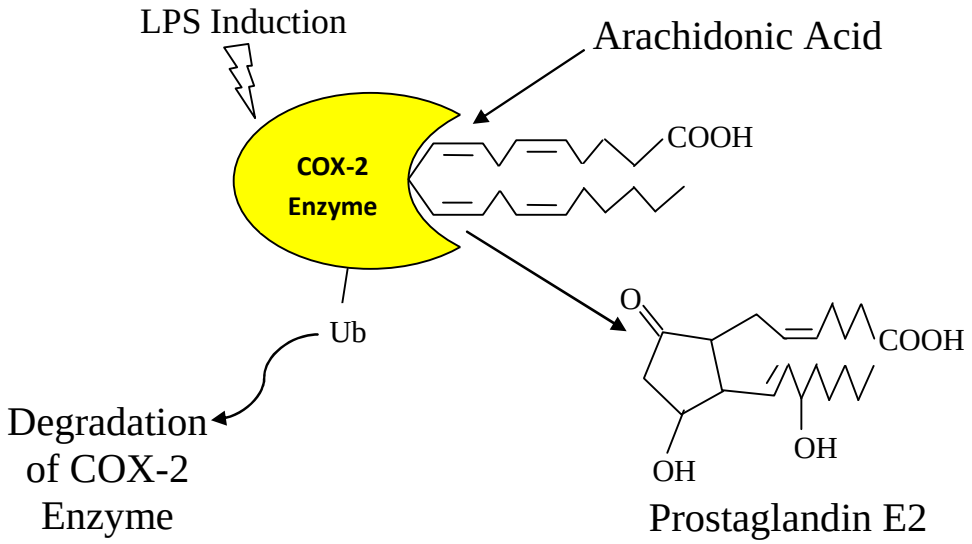
Normal Induction of PGE_2 Production by Lipopolysaccharide:



Model for the Inhibition of PGE_2 Production and Increase in Protein Levels of COX-2 After *Echinacea* Treatment:

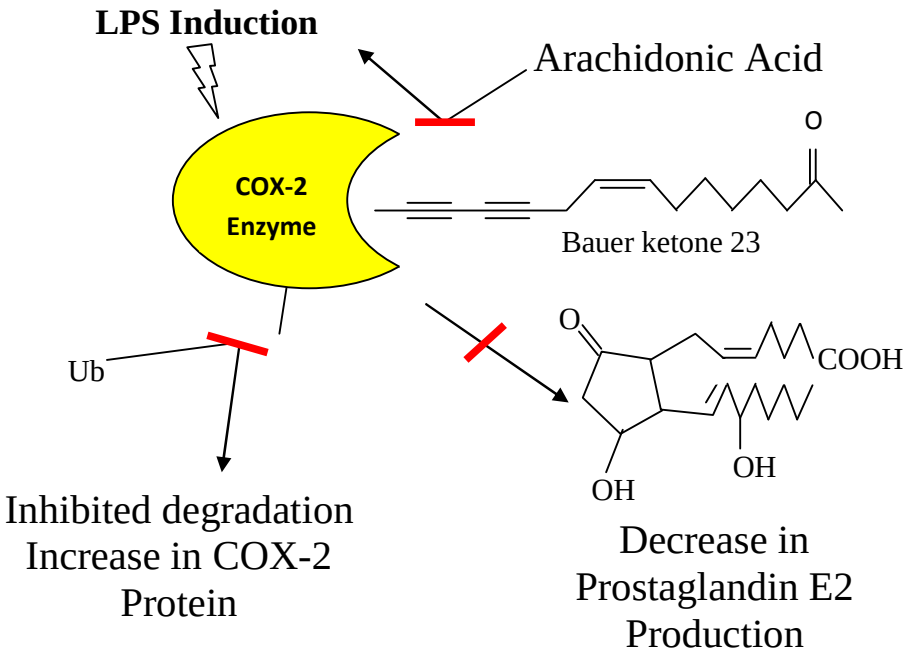


Figure 1. Model for the inhibition of PGE_2 production. Top diagram illustrates normal circumstances. Bottom diagram illustrates Bauer ketone 23 treated circumstances, which could also be replaced with the *Echinacea angustifolia* fraction 3. Ub = Ubiquitin. Red bar represents an inhibition caused by the *Echinacea* constituent or fraction. Not drawn to scale.

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they would not be targeted for degradation.

Overall Strengths of the Studies in this Dissertation

One of the major strengths of the studies presented was the quality of plant material used for the studies. Our plant material was produced by the USDA North Central Regional Plant Introduction Station, with great care to maintain disease free plants that were grown, handled, and collected under strict protocols providing material that showed consistency in biological assays. Most studies reported in the literature focus on the three medicinal species namely *E. purpurea*, *E. angustifolia*, and *E. pallida*. Due to the resources available to us, our studies were able to examine several additional species of *Echinacea* for their anti-inflammatory potential including *E. tennesseensis*, *E. simulata*, and *E. sanguinea*.

Due to the fact that many of the compounds of *Echinacea* are not commercially available for purchase other researchers must rely on isolation of constituents through extraction and fractionation methods and therefore cannot study pure constituents and their ability to modulate bioactivities. Our studies utilized pure chemically synthesized alkylamides and ketones of *Echinacea*, which allowed us to thoroughly investigate the roles these constituents play in modulating the biological endpoints chosen without interference of other compounds. The synthetic compounds were available for producing standard curves for GC-MS quantification that allowed for the identification of compounds in extracts and fractions at concentrations present in the plants. Because of this we were able to compare and combine individual constituents at concentrations present in the fractions and look for synergy or additivity. We also had access to synthetic isomers and analogs of the known alkylamides that could be produced naturally in the plant, to look at the effect of adding an extra carbon or subtracting a double bond from Bauer alkylamide 12 and assaying PGE_2 production. It was determined that through the addition of an extra carbon to the fatty acid like tail of Bauer alkylamide 12 (Bauer alkylamide 12A) a significant reduction in PGE_2

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production was achievable at a lower concentration than identified with Bauer alkylamide 12 *(8)*. Evidence that all synthetic constituents produced were in the same conformation as those present in the *Echinacea* plant was attained through NMR and GC-MS analyses.

Another strength of our approach was that we attempted to identify the lowest concentrations of extract, fraction, or pure constituent possible that were capable of significant inhibition of PGE_2 or NO production. This allowed us to use concentrations of constituents that were consistent with what was determined to be present in selected *Echinacea* fractions by GC-MS analysis. Much of the data available in the literature uses very high quantities of extract or constituent to modulate specific bioactivities, which may not be relevant to what is actually found in *Echinacea* plant preparations.

We used the RAW264.7 mouse macrophage cell model for all bioactivity assays. This cell model is strongly established in the literature for inflammatory studies and provided an excellent screening tool for identifying *Echinacea* extracts, fractions, and compounds with anti-inflammatory potential.

Limitations of the Studies

One of the major weaknesses was the use of one cell line for all studies. Although the RAW264.7 mouse macrophage cell line was a good choice for identifying *Echinacea* products with anti-inflammatory properties, it does not predict how these products might act *in vivo*. We focused on only one cell type in the inflammatory reaction therefore simplifying our results by not accounting for the other interactions that occur between cells. To further the knowledge of identified bioactive constituents from *Echinacea*, other cell lines or animal models would be necessary for studies looking at inflammatory endpoints. Without knowing the bioavailability status of the constituents when treated to the RAW264.7 cells, it is hard to draw concrete conclusions as to what concentrations of constituents are necessary to elicit the desired effect. More studies on bioavailability are necessary to better understand

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physiologically relevant concentrations of *Echinacea* extracts and constituents. Literature related to bioavailability studies with *Echinacea* extracts and constituents can be found in Chapter 2 of this dissertation.

Prostaglandin E2 and nitric oxide production were the two inflammatory endpoints chosen for screening *Echinacea* products for anti-inflammatory activity in our studies. We realize that other endpoints would be necessary to fully understand the inflammatory response of the RAW264.7 and that our conclusions are limited by relying on two inflammatory endpoints. Ongoing studies are utilizing multiplex technology to also examine a panel of 32 cytokines/chemokines, which will give a broader look at the inflammatory response and allow the studies presented in this dissertation to integrate with studies on the effect of *Echinacea* products on influenza.

Researchers studying the botanical *Echinacea* have limited access to chemically synthesized commercially available *Echinacea* constituents. For our studies we were able to use synthetic alkylamides and ketones to delve into the particular roles of these constituents in the modulation of PGE_2 and NO production, and attempt to delineate their mechanisms of action. We did not have available to us all of the alkylamides or ketones present in the plant, not to mention that there are several unknown compounds that have not been identified in the complex mixture of an *Echinacea* extract or fraction. Therefore, a limitation would be that with all of our studies there could be other, either unknown or unavailable constituents, present that are able to explain the bioactivities that were analyzed in this dissertation. In order to obtain the strongest data possible we worked diligently to isolate compounds present in the greatest quantity from the most bioactive *Echinacea* species extracts and target those compounds for synthesis.

A difficulty that we have encountered with our studies was that of stability. Our studies determined that over time, *E. angustifolia* fractions that were previously capable of

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significant PGE_2 inhibition lost their ability to inhibit this endpoint *(8)*. We had also determined that the concentrations of major alkylamides and ketones in the fraction were not changing, indicating an important role for unidentified constituents. To address issues of instability we aliquoted *Echinacea* samples into several glass vials in order to reduce the number of freeze-thaw cycles when performing bioactivity assays. Also, to assure stability of *Echinacea* fractions and synthetic constituents we froze samples at -80 °C under argon gas.

A limitation of the studies conducted in this dissertation that must be acknowledged is that all of our studies were conducted on the root material from *Echinacea*, therefore it is possible that preparations from aerial parts of the *Echinacea* plant could produce significantly different data than what we have observed, although several of the constituents found in the root material are also present in the aerial parts *(9)*. Another limitation was that a Soxhlet method of extraction was used to prepare all species extracts, including those used for fractionation. Due to the aggressive nature of this extraction procedure, which includes high heating temperatures, it is possible that certain compounds were destroyed or altered to a non-natural state. These non-natural compounds could have had an effect on our bioassays, perhaps accounting for the inability of Bauer alkylamide 11 to explain a majority of the PGE_2 inhibition capabilities identified with the third round *E. angustifolia* fraction 3E40, where alkylamide 11 made up approximately 96% of the fraction by dry weight. In order to circumvent the possibility of potentially creating non-natural compounds, perhaps a more gentle method of extraction could be utilized.

The method of bioactivity-guided fractionation allows for the separation of constituents in *Echinacea* extracts to identify which compounds may be important for noted bioactivity. This method provides a limitation in that when fractionation and therefore separation of constituents occurs, these divisions could potentially separate constituents that

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must be present together to illicit a specific response. For example because we used semi-preparative reverse-phased HPLC for fractionation which separates constituents in the extract based on hydrophobic properties, perhaps the phenolics that come out in the earlier fractions and the alkylamides which come out in later fractions could act as stronger inhibitors of PGE_2/NO/TNF-α production had they not been separated in different fractions.

Follow-up Studies

The initial results we obtained from our *Echinacea* species extract data indicate that *E. sanguinea* was the most active species measured via the inhibition of PGE_2 production. It would be interesting to examine this alkylamide rich species further to identify key constituents leading to the noted bioactivity through fractionation studies.

From our qRT-PCR time course studies it was determined that *E. angustifolia* fraction 3, enriched fraction 3, the combination of Bauer alkylamide 11 and Bauer ketone 23, and these constituents individually coincubated with LPS significantly inhibited TNF-α gene expression at 24 hours compared to the media + DMSO + LPS control in the RAW264.7 cells. The literature on TNF-α and *Echinacea* suggests that alkylamides bind cannabinoid receptor 2 to mediate the modulation of TNF-α at nanomolar concentrations, through the activation of cAMP, p38/MAPK, JNK, and NF-κB signaling *(10)*. It would be interesting to use qRT-PCR to examine treatments listed above after using a cannabinoid receptor 2 antagonists (SR144528) to determine if this receptor plays a role in the TNF-α modulation we identified. Cannabinoid receptor 2 has been identified in the RAW264.7 mouse macrophage cells induced with 100ng/ml LPS and cannabinoid receptor 1 was not detected (*11*). Pathways important for the inhibition of TNF-α gene expression could be analyzed using specific synthetic inhibitors such as, PD98059 for MAPK/MEKK, U0126 for MEK1/2, SB203580 and SB202190 for p38/MAPK, SP600125 for JNK, and parthenolide for NF-κB. Our studies showed that the *Echinacea* treatments inhibited gene expression of TNF-α after a

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24 hr treatment and therefore this time point would be a relevant starting point for conducting studies with receptor and signaling inhibitors. The microarray study conducted in this dissertation examined gene expression after an 8 hr incubation of *Echinacea angustifolia* samples with the RAW264.7 cells, consistent with the time point where PGE_2 production was analyzed. From our qRT-PCR studies it would appear that the more informative time point may be 24 hr treatments for the microarray. Therefore, we would propose that future microarray studies dealing with *Echinacea* treatments in the RAW264.7 cell model be carried out after a 24 hr treatment. Information gathered from microarray study at the 24 hr time point would also be relevant for the elucidation of pathways important for the inhibition of NO and TNF-α.

We determined that the combination of synthetic Bauer alkylamide 11 and Bauer ketone 23, as well as these constituents individually were capable of significant inhibition of nitric oxide production, whereas the *E. angustifolia* fraction and enriched fraction were not. We also determined that the enriched fraction was increasing the mRNA levels of iNOS, whereas the fraction and constituents had no effect. Also, the iNOS western blot data indicates that the enriched fraction, combined constituents, nor the constituents individually affected the protein levels. To better understand how these *Echinacea* treatments are inhibiting NO production we would propose looking at iNOS activity levels in the RAW264.7 cells after treatment with LPS. Nitric oxide is produced through the action of nitric oxide synthases through the conversation of L-arginine to L-citruline *(12)*. Nitric oxide acts as a signaling molecule and a free radical that can react with other free radicals to form more potent radicals like peroxynitrite and other lipid peroxides and has been shown to be involved in the inflammatory response of macrophages. We would propose parallel studies to analyze the effect of *Echinacea* treatments on arginase enzyme, which converts L-arginine

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into L-ornithine leading the way to the production of polyamines and L-proline that is involved in cell growth and wound healing.

Previously in this chapter a model was proposed to explain the action of *Echinacea* treatments to inhibit COX-2 activity and therefore PGE_2 production, as well as to explain the induction in COX-2 protein identified after treatments of *E. angustifolia* fraction or Bauer ketone 23 to the LPS induced RAW264.7 cells. The active site of the COX-2 enzyme contains specific residues, specifically Arg-120, Tyr-355, and Glu-524 *(13)*. Predictive computer modeling of the COX-2 enzyme could provide information as to whether compounds of *Echinacea* have the potential to bind to the active site of this enzyme stably and therefore compete with arachidonic acid for the binding site (Insight® II version 97.0). This modeling could also show possible ubiquitination sites and predict whether *Echinacea* compounds could block these sites. After verification of possible interactions of the *Echinacea* constituents with the COX-2 enzyme, biological assays could examine the proposed competition between arachidonic acid and *Echinacea* fractions/constituents for the active site of COX-2 through competitive binding assays. To assess ubiquitination status of COX-2 enzyme we propose using recombinant COX-2 protein and co-transfect the RAW264.7 mouse macrophage cells with Flag-COX-2 and His-Xpress-Ub. Flag-COX-2-Ub conjugates could be pulled down with Flag-beads and detected by western blot analysis using anti-Xpress antibody. In general, isolation of the COX isoforms, which are integral membrane proteins, have been successful using microsomal preparations from insect cells expressing recombinant protein using detergent solubilization followed by purification to yield an apo-enzyme that can be reconstituted with hematin (*14*). Successful His tagging methods have lead to the purification of active native COX enzymes.

Overall Conclusions

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The data presented in this dissertation advances the knowledge and understanding of the anti-inflammatory potential of the botanical *Echinacea* and due to the simplicity of the model chosen for these studies can be used as an initial study to guide future research and development of *Echinacea* products for immune modulation. All together, these studies draw attention to the complex nature of studying botanical supplements and the care that must be taken to identify key components relevant to specific bioactivities. The endpoints chosen for screening, PGE_2 and NO, play broad roles in the inflammatory response for many disease states and therefore our research does not target a specific disease but remains in context with how *Echinacea* is utilized by consumers for the treatment of cold or upper respiratory tract infections.

As stated in chapter one of this dissertation the hypothesis that drove the direction of this research was that *Echinacea* extracts, fractions, and pure constituents, such as alkylamides and ketones, are capable of inhibiting prostaglandin E2 production through the inhibition of the NF-kB signal transduction pathway, which therefore inhibits the expression of COX-2 in a RAW264.7 mouse macrophage model. Our mechanistic studies disproved this hypothesis, indicating that the identified inhibition of PGE_2 production after treatment with *Echinacea* fractions and selected constituents in LPS induced RAW264.7 cells was due to an inhibition of COX-2 activity, not acting at the gene expression level. Therefore it is unlikely that NF-κB signaling events are disrupted by *Echinacea* products to inhibit PGE_2. Initially we proposed that the NO inhibition caused by the *Echinacea* products was also due to the inhibition of NF-kB signal transduction leading to an inhibition of iNOS gene expression, but it appears that from our qRT-PCR data that this is most likely not the case. We determined that the combination of Bauer alkylamide 11 and Bauer ketone 23, as well as each constituent individually was able to significantly inhibit NO production, but there was no effect on iNOS gene expression over a 24 hr time course, or iNOS protein levels at an 8 hr

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time point. Therefore, iNOS activity may be the target of these treatments and follow-up studies addressing this issue will need to be conducted.

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